The affective response to CO2 in healthy volunteers : an instance of a primal emotion

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*Cover***:** The dawn of man. Drawing based on a scene of the movie '2001: A Space Odyssey' (1968).

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per sara,

per mio padre

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Contents

Introduction

Aim of the thesis

The inhalation of high concentrations of carbon dioxide (CO_2) triggers a powerful instinctual response in humans characterized by the rapid occurrence of an intense urge for air and a sudden increase in ventilation. The aim of the present thesis is to experimentally characterize the emotional response to inhalation of CO_2 in humans.

In experimental settings, it is possible to administer CO_2 to human subjects in a safe, standardized, and reliable manner. By studying the subjective response to CO_2 in a laboratory context, it is possible to experimentally reproduce and characterize the emotional response associated to a basic instinct for life, such as air hunger. That response represents an instance of a "primal" emotion.

This experimental paradigm provides researchers in affective neuroscience and experimental psychopathology with an important opportunity to understand how affective responses rooted in instinctual behaviours may inform about the psychopathological states observed in psychiatric disorders. In fact, there have been theories suggesting that panic attacks originate from a phylogenetically ancient, innate, instinctual response aimed to protect the organism against asphyxia. In the studies reported in the present thesis, we analyze the phenomenology and pharmacology of CO_2-induced emotion, and we test the hypothesis that the instinctual responses to CO_2 inhalation in healthy human subjects share closely related features with panic attacks.

Background

Instincts are inherent inclinations of an organism toward a particular behaviour with high survival value. In animals, as well as in humans, when strongly aroused, instincts incorporate an imperious sensation and a compelling specific intention. The subjective element that coalesces these physiologic entities has been defined as a primal (or primordial) emotion by the physiologist Derek Denton (Denton, 2006). Indeed, instincts and emotions are closely bound. William James (James, 1918) stated that ''in speaking of instincts, it has been

impossible to keep them separate from the emotional excitements which go with them […] They shade imperceptively into each other".

Primal emotions, such as hunger for air, hunger for minerals or for food, thirst, pain, or sexual arousal, are all generated by vegetative systems and dictated by chemical events sensed by interoceptors. When aroused, these emotions completely occupy the stream of the consciousness. They serve a homeostatic function and are genetically programmed to guard the physicochemical constancy of the internal environment of the body (Denton, 2006).

Air hunger is a form of primal emotion, programmed to protect the organism against the risk of impending suffocation and acidosis, and aimed to restore the acid-base balance and oxygen supply by cueing breathing (Liotti *et al*, 2001). Air hunger is characterized by the conscious perception of an urge to breathe, which arises when pulmonary ventilation is insufficient for the metabolic needs of the organism (Lansing *et al*, 2009). Air hunger, specifically, is believed to result from a mismatch of afferent information between respiratory drive and ventilation, and is therefore dependent on afferents originating from brainstem, informing about the automatic drive to breathe and related motor activity, and afferents from lungs, informing about the amount of pulmonary ventilation (Lansing *et al*, 2009).

Different interoceptors are involved in the origin of air hunger, including central and peripheral CO_2/H^+ chemoceptors, which project to brainstem nuclei, mechanoreceptors of the chest wall, and a number of pulmonary receptors such as vagal receptors, stretch receptors, type-J receptors in the walls of alveoli and capillaries, and irritant receptors in the upper airways and the tracheobronchial walls (von Leupoldt and Dahme, 2005). A classic stimulus to induce air hunger is the administration of CO_2-enriched air mixtures; however other ventilatory stimuli, such as hypoxia and metabolic acidosis, can trigger air hunger by stimulating the brainstem reflex ventilatory drive. The level of air hunger is related to the level of ventilatory drive irrespective of the ventilatory stimulus and regardless of whether this arises from central or peripheral chemoreceptor afferent activity, suggesting that air hunger is directly dependent on the reflex respiratory drive (Moosavi *et al*, 2003). Although it is not the only stimulus capable to induce air hunger, acute hypercapnia per se is a

powerful way to evoke air hunger, even in absence of the afferent contribution from respiratory muscles, as demonstrated by an experiment showing that totally curarized, and mechanically ventilated, human subjects reported severe air hunger during hypercapnia (Banzett *et al*, 1990).

In experimental psychiatry, the acute administration of CO_2 has been widely used for more than 25 years as tool to experimentally reproduce panic attacks in patients affected by Panic Disorder (PD) (Griez and Schruers, 1998). CO_2-challenges are relatively easy and non-invasive procedures, the two most common techniques of CO_2 administration consisting in either a single or double vital capacity inhalation of 35% CO_2 or 5-20 minute inhalation of 5-7.5% CO_2 through continuous exposure (Verburg *et al*, 2001). The assessments of the subjective response among the studies have classically used self-report measures based on the physiological and psychological panic symptoms defined by the Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV-TR) criteria for panic attack (Verburg *et al*, 2001).

The interest of researchers in experimental anxiety in the panicogenic properties of CO_2 arose from the well-established finding that a proportion of patients affected by PD are hypersensitive to CO_2, relative to healthy individuals and patients suffering from other psychopathological conditions (Griez *et al*, 1990a). When PD patients undergo a CO_2-challenge, they experience an emotion closely resembling the panic attacks that occur naturally, out of the blue, as a consequence of their condition. First-degree relatives of PD patients share, to a lesser extent, an increased sensitivity to CO_2 (van Beek and Griez, 2000). These findings have indicated that the vulnerability to CO_2 could represent a genetic trait marker of panic susceptibility, and stimulated the use of the CO_2-challenge as an experimental model of panic, eventually contributing to the formulation of several influential theories of the etiology of PD.

One of these theories was proposed by Donald Klein in 1993 (Klein, 1993; Preter and Klein, 2008), based on the hypersensitivity to CO_2 observed in PD patients, and accounted for a large number of experimental findings. He proposed that panic originates from the maladaptive response of a deranged inborn suffocation detector, sensitive to CO_2. When the

suffocation monitor erroneously detects excessively increased CO_2 concentrations, it alerts the organism to the threat of impending asphyxia, eventually leading to a behavioural and emotional response characterized by an urge for breathing and acute panic. Klein, in other words, proposed that panic attacks were false alarms of suffocation, occurring in individuals with a dysfunctional CO_2 suffocation detector (Klein, 1993; Preter *et al*, 2008).

This theory postulates the existence of an innate suffocation detector as part of the physiologic repertoire of human organism. The idea that panic attacks originate from a hard-wired, innate, instinctual response programmed to protect the organism against asphyxia fits perfectly with the concept of primal emotions previously described. The feeling of panic would correspond to the subjective feeling of air hunger, which completely occupies the stream of consciousness when CO_2 levels are excessively raised. If the hypothesis is true, every individual with no vulnerability to panic would experience a panic-like feeling when exposed to sufficiently high CO_2 doses. The CO_2-induced subjective response should be comparable to clinical panic, both in terms of phenomenological features and pharmacological modulation. Furthermore, the panic-like feeling evoked by CO_2 should closely correlate with the feeling of air hunger.

Based on these assumptions, the objective of the present thesis is to investigate the response to exposure to high-doses of CO_2 in healthy volunteers, aiming to answer the following research questions:

1. Does CO_2 induce acute emotional distress, resembling panic, in healthy volunteers when CO_2 doses are adequately increased?
2. Is the phenomenology of response to CO_2 similar in healthy subjects and patients with PD?
3. Is the emotional response to CO_2 specifically associated to respiratory sensations?
4. Does the manipulation of the serotonin system, which modulates clinical panic, influence the response to CO_2?

Finally, we try to answer the question: Does CO_2 really evoke a true emotion?

Outline

In chapter 1 we present a comprehensive review of the literature on the human response to exposure of exogenous CO_2 and its psychopharmacological properties. In the first half of the 20th century, CO_2 has been used as an anaesthetic and therapeutic agent, while in the last 25 years it became widely used in experimental psychiatric research as an experimental model of panic. Early human physiology studies and reports of cases of CO_2 poisoning and intoxications provide clues of the subjective effects of breathing CO_2 at different concentrations in humans, and indicate that the prolonged exposure at doses higher than 10% is associated with an intense emotional distress. The review also provides a complete summary of the studies on the genetics and pharmacology of the response to CO_2 in humans and animals, and includes an overview of the main mechanisms underlying CO_2-induced affective responses. It concludes with the evolutionary-inspired hypothesis that CO_2 might act as an agent of a primal emotion serving a homeostatic function, in the control of respiration and acid-base balance.

Due to ethical and methodological limits, it's not possible to test the affective response to prolonged exposure to CO_2 at doses higher than those used in the above-mentioned early physiology studies. However, documenting a dose-response relationship using very short exposures to CO_2 may provide a safe and easier solution to extrapolate information about the response to extreme concentrations. In the study reported in chapter 2, we investigated the acute emotional response to increasing concentration of CO_2 in 64 healthy subjects, who underwent a double inhalation of four gas mixtures containing respectively 0, 9, 17.5 and 35% CO_2 in a cross-over, randomized design. We demonstrated that CO_2 challenges induced a dose dependent negative affect compliant with the DSM-IV definition of panic attack. Approximately one third of the subjects experienced a short-lived panic attack with the highest CO_2 concentration. These data indicate that CO_2 induces acute emotional distress, resembling panic, in healthy volunteers when CO_2 doses are adequately increased.

To investigate the phenomenology of CO_2-induced emotional response, in chapter 3 we report the results of a factor analysis study of the CO_2 challenge-induced panic symptoms, using 4 different CO_2 concentrations. Three clusters of symptoms were extracted:

respiratory, cognitive, and neurovegetative, which correspond closely to the clusters of symptoms occurring in PD patients during their spontaneous attacks. Furthermore, the respiratory symptoms induced by CO_2 were the best correlate of the emotional distress evoked by the challenge at the highest doses. These findings underlie the specificity of respiratory symptoms in the phenomenology of the emotional response to CO_2, and fit numerous epidemiological and clinical evidences indicating that panic and respiration are closely related.

In chapter 4, we characterized the emotional response to CO_2 using a multidimensional instrument that measures the sensorial and affective components of respiratory discomfort. All the dimensions of respiratory discomfort were affected by the challenge. We found that the rating of respiratory discomfort was strongly correlated to the intensity of the CO_2 – induced panic feeling, showing a higher predictive power relative to that of all non-respiratory DSM-IV panic symptoms, and was able to accurately discriminate between responders and non-responders to the CO_2 challenge. By demonstrating that a scale rating respiratory discomfort could be successfully applied as a tool to evaluate the subjective response to CO_2, we provided support to the idea that respiratory symptoms are a central phenomenon in experimental panic induced by CO_2.

It has been reported that in PD the manipulation of serotonin precursor tryptophan alters the vulnerability to experimentally induced panic. In the study reported in chapter 5, we aimed to investigate the effects of tryptophan depletion and tryptophan loading on CO_2induced panic response in healthy volunteers. We found that CO_2-induced subjective distress and air hunger were significantly lower after tryptophan depletion, and that the subjective distress was positively correlated to the availability of serotonin precursors. These findings go in the opposite direction relative to the results observed in PD patients; however they are in line with preclinical data indicating a role for the serotonergic system in promoting the aversive respiratory sensations to hypercapnic stimuli (Severson *et al*, 2003). The differences observed in our study, compared to previous findings in PD patients, might depend on an altered serotonergic modulatory function in PD patients compared to healthy subjects.

In chapter 6, we apply the criteria proposed by Cabanac for the definition of emotion to the findings of our studies, in order to test if the subjective response to CO_2 truly complies with the definition of emotion. Finally, we discuss other influential authors' views on primal emotions. To conclude, we summarize our findings and suggest future directions for further research.

Chapter I

On the psychotropic effects of Carbon Dioxide

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Abstract

It has been well established that the inhalation of Carbon Dioxide (CO_2) can induce in humans an emotion closely replicating spontaneous panic attacks, as defined by current psychiatry nosology. The purpose of this review is to provide a critical summary of the data regarding CO_2's psychopharmacological properties and underlying mechanisms. The authors review the literature on the human and animal response for the exposure of exogenous CO_2 focusing on five points of interest: 1) the early history of the use of CO_2 as an anesthetic and therapeutic agent, 2) the subjective effects of breathing CO_2 at different concentrations in humans, 3) the use of CO_2 in experimental psychiatric research as an experimental model of panic, 4) the pharmacological modulation of CO_2-induced responses, and 5) the putative neurobiological mechanisms underlying the affective state induced by CO_2. The authors conclude with an evolutionary-inspired notion that CO_2 might act as an agent of a primal emotion serving a homeostatic function, in the control of respiration and acid-base balance.

Keywords

Carbon dioxide; panic disorder; anxiety; fear; primal emotion; CO_2 challenge; respiration; human models.

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1. CO_2 from an early anaesthetic agent to an experimental tool in anxiety research

First known references to what is now called carbon dioxide (CO_2) originated by the work of Helmont (1577-1644), who identified CO_2 and Black (1728-1799), who isolated CO_2 from chalk by acid and established that was identical to the air produced in animal respiration and that could have toxic effects (Davison, 1965; Foregger, 1957). In 1777, Lavoisier (1743–1794) proposed that inhaled oxygen (O_2) was transformed in the body to CO_2 and that respired air, containing low levels of O_2 and increased CO_2 concentration, was unfit for rebreathing. The first experiment shedding light on the effects derived from inhaling CO_2 was carried out by Hickman (1800-1830), who observed that putting small animals under a glass bell, they became initially restless and breathing hard until respiration arrested. Immediately thereafter, the experimenter could cut the animals' ear without they were showing any sign of pain. After the operation the animals recovered. Hickman suggested that the unconsciousness induced by inhalation of CO_2 to produce what he called "suspended animation" could be used to provide pain-free surgical procedures (Hickman, 1965). The anesthetic properties of CO_2 were formally recognized only a century later, in the early XX century. When anesthesia began to be accepted as a part of the surgical routine, the technique employed was generally asphyxial rebreathing. Anesthetic procedures were brief and patients usually were able to tolerate the asphyxial assaults (Morris, 2002). Leake and Waters (1929) studied the effects of mixtures of CO_2 with O_2 on rabbits and dogs, and performed some preliminary studies on man; they confirmed Hickman's reports and supported his statement that CO_2 produced unconsciousness. The same authors (Leake, 1929; Loevenhart, 1929) separately participated in administration of repeated inhalations from 10 to 40% CO_2 to psychiatric patients as a form of shock therapy in treatment of "dementia precox", "manic depressive insanity", and "involutional melancholia", obtaining striking effects in patients who had been mute or inaccessible for years. The results were attributed to CO_2-induced cerebral stimulation. The positive effects however were short-lived and disappeared within 2 to 25 minutes following the inhalations (Loevenhart, 1929).

Even if the interest of research in its anxiogenic properties raised only in the last 25 years (see section 3), CO_2 was shown to be eliciting anxiety in vulnerable individuals since 1919, when Drury showed that CO_2 caused CO_2-induced hyperventilation and feeling of breathlessness in soldiers affected by the irritable heart syndrome, an early diagnostic entity similar to Panic Disorder (PD), at a lower inspired CO_2 concentrations than in control subjects. The finding was replicated 30 years later in patients with anxiety neurosis, when it was shown that the prolonged rebreathing of CO_2–enriched gas mixtures induced symptoms resembling those of their anxiety, particularly during so-called "anxiety attacks" (Cohen and White, 1951).

In spite of these early suggestions, the psychotropic properties of CO_2 remained almost unrecognised until the middle eighties. For almost seventy years since Drury's reports, the use of CO_2 inhalation in psychiatry has been confined to rare and peculiar therapeutic techniques used in the 1950s as the Meduna's "Carbon Dioxide therapy" and La Verne's "Rapid coma technique" (Laverne, 1953; Meduna, 1955). These authors described the use of prolonged exposure to very high concentration of CO_2, respectively mixtures containing 30% and 70% CO_2 in patients with conversion symptoms, anxiety neurosis, personality disorders, and addiction disorders. The rational of the techniques is still unclear. The treatment was based on an organic theory claiming that "neurosis" was caused by abnormally low threshold of stimulation and CO_2 treatment was intended to raise the threshold of stimulation to the normal level of resistance to noxious stimuli. Meduna's technique consisted in repeated inhalations of 30% CO_2 -70% O_2 until induction of coma, while La Verne used 70% CO_2 in order to obtain a more rapid loss of consciousness. During CO_2 therapy subjects lost consciousness after 8-10 deep breaths of 30% CO_2, but in some cases forced administration of CO_2 was continued until 80 inhalations. During the initial phase the patient experienced a large increase in respiration rate and in respiration volume, a sense of panicky breathlessness, flushing of the face, and perspiration. Many patients felt that they were choking and raised their hands to remove the mask or to turn their heads. Some patients fought violently, kicked and screamed at top of their voices. Obviously the first complication was the fact that it could reinforce anxiety in patients with overt anxiety and in those who fear the treatment, and the reinforcement of anxiety was usually proportionate to the length

of the treatment. In the assessment of the psychodynamic factors responsible of the effectiveness of the treatment, it was stated that CO_2 inhalation definitely provoked feelings of smothering and of a literal threat to life, thus causing a stress situation which required an active effort to be handled (Rogers, 1955).

In the late '50s, CO_2 was turned into possible adjunct to psychiatric treatment based on the behaviorist concept of "reciprocal inhibition", which refers to the complete or partial suppression of anxiety responses to specific stimuli as a consequence of the immediate evocation of other responses physiologically antagonistic to anxiety. The technique, proposed by Wolpe (Wolpe, 1958), aimed to condition a new behavioral response, evoked by CO_2, which would have been able to replace the anxiety response. CO_2 was used for this purpose, and 20 cases of pervasive anxiety successfully treated by CO_2 were reported. After Wolpe's seminal work, other experiments were carried out to test the assumption that CO_2 inhalation has such an anxiolytic effect. Although these reports were consistent with the earlier findings, indicating that CO_2 reduced anxiety, refined replications of those studies by Griez & van den Hout in the early '80s repeatedly indicated that inspiring CO_2 in itself was not anxiety-reducing at all (Griez and van den Hout, 1982; van den Hout and Griez, 1982).

A different use of CO_2 inhalation was made by Orwin (Orwin, 1973) who treated situational phobic patients with the so-called technique "respiratory relief". CO_2 mixtures were introduced because of their properties of respiratory stimulation: it was believed that intensifying the need to breathe would have resulted in an augmented respiratory relief. The basic hypothesis was that a respiratory relief, satisfying an intense need to breathe, would inhibit the degree of anxiety provoked by the simultaneous presentation of the phobic stimulus.

Shortly after these preliminary suggestions, the interest of anxiety researchers in CO_2 began to shift from its putative anxiolytic properties to the current use of CO_2 as a panicogenic agent (see section 3).

2. Subjective and objective effects of CO_2 in healthy humans

CO_2 is the fourth most abundant gas in the earth's atmosphere. It is a colorless, odorless, non-irritating gas present in normal air at the concentration of 0.027-0.036%. It is faintly acidic-tasting and non-flammable at room temperature. CO_2 is formed during metabolism in humans and animals, in fermentation and decomposition and during the burning of fossil fuels. It is also available in solid and liquid forms.

Short exposures to concentrations lower than 2% have no noticeable effects on man. The symptoms depend on the concentration and the period of the exposure. Up to 4-6% CO_2 the only effects are a marked increase in ventilation, and mild dizziness and headache. At 6% of CO_2 dyspnea appears, the respiration can be difficult, distressing and increasingly anguished for some individuals. Then tachycardia, asthenia, visual disturbances, worsening of headache, dizziness, and confusion may occur. At 8% dyspnea becomes severe (Arena and Drew, 1986). The effects become more marked when at least 10% of the gas is present in the air. Tinnitus, tremor, profuse perspiration, increased blood pressure, abnormal eye movements can occur. Some individuals cannot tolerate concentrations above 10% for more than 5-10 minutes. Others can support it much better and there are cases of 1 hour exposure at concentrations near 10% CO_2 without evident harm (De Coninck, 1963). After prolonged exposures to 12% CO_2 dyspnea is extreme, accompanied by drowsiness, muscle twitching, vomit, photophobia and retinal degeneration. Prolonged exposures (more than 5 minutes) to higher concentrations (above 17%) result in CO_2 intoxications, characterized by stupor, loss of consciousness, convulsions, and eventually death.

In this section, we report the effects of different doses of CO_2, derived from accidental or occupational exposures and experimental procedures. In reviewing the literature we pay attention to any clue for anxiety, panic and extreme discomfort caused by breathing CO_2. We focus on the behavioural and psychological dimension and try to extrapolate data about these dimensions if they are not explicitly investigated in the studies.

2.1 Psychotropic effects of CO_2 following accidental exposure

2.1.1 CO_2 poisoning

Cases of CO_2 poisoning are described in the literature (Langford, 2005). Massive CO_2 poisoning occurred in occasion of two catastrophic events in 1984 and 1986, both occurred in Cameroon, Africa, when emissions up to 10^9 m^3 of gases were released from volcanic lakes. It was concluded that the gas was nearly all CO_2 and the poisonings could be attributed to it (Kling *et al*, 1987). Exogenous CO_2 may also be recovered from lime or cement kilns, flue gases, fermentation processes, and natural gas wells. It is also used for preserving foods, especially during transportations. Exposures may occur in mines, caves, tunnels, wells, holds of ships, tanks or any place where fermentation processes have formed CO_2. The use of dry ice and CO_2 fire extinguishers also offers exposures. A US environmental Protection Agency study investigated deaths resulting from the discharge of CO_2 fire extinguishing systems between 1975 and 2000; a total of 51 incidents occurred, with 72 deaths and 145 injuries (EPA, 2000). The course of the acute poisoning described in case reports (Gill *et al*, 2002; Romeo *et al*, 2002; Troisi, 1957; Williams, 1958) consists in a rapid loss of consciousness accompanied by minor symptoms as giddiness, pain in the legs, and pain and dryness of the throat. In these cases it was concluded that the percentage of CO_2 in the inspired air could have been between 15% and 30%. In an interesting report (Kreiss *et al*, 2003), a man and a woman reported episodic shortness of breath, confusion, poor concentration, light-headedness, dizziness, headache, blurry vision and fatigue every time they went down in the basement of their house. Their symptoms always resolved within minutes of returning upstairs, however basement-related symptoms persisted for two years, until the woman went to a hospital emergency department on two consecutive mornings with shortness of breath, rapid heart rate, and panic. The air sampled in the basement showed CO_2 concentrations of 10-12%.

Another example of CO_2 hazard is in general aviation, where CO_2 is used in fire-extinguishing system and in form of dry ice. Cases have been reported where firing of false alarms caused releases of CO_2 in the aircraft, resulting in partial incapacitation of the crew and eventually crashes. In a review (Gibbons, 1977) of CO_2 hazards in general aviation, it is

stated that CO_2 can have definite adverse effect on passengers and flight crew, and that possible panic, and associated performance decrements, that might occur from sudden, rapid, uncontrollable breathing could also lead to an aircraft accident.

2.1.2 CO_2 and panic hazards in scuba divers

CO_2 build-up constitutes a significant potential hazard which may lead to diving accidents and fatalities (Edmonds, 1992). CO_2 can accumulate insidiously in the diver, who intentionally holds the breath intermittently (skip breathing) in a mistaken attempt to conserve air. Moreover, divers may hypoventilate unintentionally if a tight wetsuit or buoyancy compensator jacket restricts chest wall expansion, or if ventilation is inadequate in response to physical exertion as when swimming against a strong current. Strenuous exercise can increase the rate of CO_2 production by more than 10-fold, resulting in a transient elevation in arterial CO_2 pressure (Pa CO_2) of more than 60 mm Hg. Furthermore, increased gas density at depth increases the work of breathing and thus the effort required to vent accumulated CO_2. When diving with any closed or semi-closed circuit rebreathing apparatus, part or all of the diver's exhaled breathing gas is recirculated and rebreathed after CO_2 is removed via a CO_2-absorbent material. Inadequate CO_2 absorption is possible with improper packing or saturation of the absorbent (Edmonds, 1992). A further risk occurs when the diver who runs out of air, removes the regulator, and takes repeated breaths from a trapped gas pocket that has formed under the roof of a cave, then rapidly developing hypercapnia.

In more than 60% of scuba diving fatalities, the cause is listed as drowning. Drowning is usually caused by specific problems, as lack of air, entanglement, air embolism, narcosis, and panic. There is considerable evidence that the occurrence of panic contribute to a substantial number of diving accidents and fatalities (Morgan, 1995). Air embolism, a common cause of diving fatalities, may also result from a rapid ascent due to panic (Sweeney, 1993). Mortality associated with diving is not restricted to the novice divers: it has been reported that more than half of a sample of experienced divers have experienced panic or near-panic behaviour while diving on one or more occasions (Morgan, 1995). While Brown has stated that emotional instability or “panic plays a big role in many, if not most,

diving accidents" (Brown, 1982), Graver had claimed that panic "[…]is the diver's worst enemy" (Graver, 1993).

Morgan and Raven have shown that trait anxiety can accurately predict the majority of those individuals who will experience respiratory distress while wearing the Self-Contained Breathing Apparatus (SCBA) and performing heavy exercise (Morgan, 1995). According to Morgan, respiratory responses to exercise and CO_2 are influenced by psychopathology, and individuals classified as "hyperventilators" or "hypoventilators" appear to be particularly vulnerable to problems associated to the use of SCBA (Morgan, 1983). There is evidence that individuals who become certified scuba divers, score in the low range on measures of state and trait anxiety. Moreover, there is evidence that individuals who discontinue involvement in scuba diving, display significantly higher trait anxiety than those who continue (Morgan, 1995).

In summary, there is agreement that panic behaviour is one of the primary reasons for scuba diving injuries or fatalities, and there is evidence that certain individuals are more likely to panic while scuba diving than others. The idea that panic amongst divers might be a consequence of increased CO_2 is appealing, as it has been well demonstrated that CO_2 load is a frequent occurrence during scuba diving. The fact that divers have been shown to be at risk for performance impairment, immediately after a period of CO_2 retention load (Henning *et al*, 1990), provides further support to that hypothesis.

2.2 Experimental studies

Ethical issues determine a limitation in the CO_2 doses suitable for experimental studies. Up to 20 minutes of 7.5% CO_2 and a few breaths of 35% CO_2 are absolutely safe procedures, which have been used in psychiatric research for two decades without any unexpected adverse event. They will be described in chapter 3.

The effects of prolonged exposures to higher doses, as more than 7.5% CO_2, can be only extrapolated from reports of human physiology experiments. In fact, many experimental studies were conducted in the past to explore the physiological response to CO_2 in humans, testing the highest tolerable concentrations of CO_2.

In an early study, Brown (Brown, 1930) studied 7 male subjects with percentages of CO_2 ranging from 5.5% to 12.4% and O_2 ranging from 14.4% to 39.7%. He stated that these concentrations were "so excessive as to be intolerable within a comparatively short time". The exposure periods showed considerable variations: from 3 to 10 minutes with 7.5% and 8.8% of CO_2 and from 0,75 to 2 minutes with 10.4% and 12.4%. Subjective symptoms were panting, dyspnea, dizziness, sensation of flushing, sweating of the face, drowsiness, headache, sense of impending collapse, irritation of the throat and choking sensation. One subject actually collapsed at 12.4% CO_2 and other two reported the sensation of impending loss of consciousness. High O_2 compared to low O_2 did not minimize the effects. In 1946, Dripps and Comroe (Dripps and Comroe, 1947) administered inhalations of 7.6 and 10.4 % CO_2 to 44 male young subjects for a period of time ranging from 2,5 to 6 minutes at 10.4 % CO_2 and from 2.5 to 8.5 minutes at 7.6 % CO_2. The symptoms most frequently noted during inhalation of 10.4 % CO_2 were dizziness, dyspnea and faintness. In less than a half of the total of the subjects also sweating, restlessness, headache, fullness in head were reported. Other symptoms noted were: general discomfort, exhaustion, palpitation, substernal pain, muscle tremors, tingling, irritation of the nose, cold extremities, mental clouding, dimness of vision, analgesia, unawareness of the surrounding, and a sensation described "as being in the first stage of nitrous oxide anaesthesia". In 1960, in a study by Sechzer *et al*. (1960), 12 healthy male volunteers inspired concentrations of CO_2 in O_2 ranging from 7% and 14% for periods of 10- 20 minutes. Several subjects described the experience as "horrible", "unbearable", "like strangling" or "suffocating". Others experienced a fear of calamity or a feeling of impending death. Profuse sweating, restlessness, headache, chills and excitement were reported, and visual and auditory hallucinations also occurred in few cases. Some subjects vomited into the mouthpiece, others lost consciousness. In 1965, an experiment (Brackett *et al*, 1965) was performed in a large environmental chamber: 7 human volunteers were exposed to a 7% CO_2 and then to a 10% CO_2 atmosphere in a room where the CO_2 tension was automatically maintained at the desired level. All the subjects were able to tolerate the 7% CO_2 atmosphere for as long as it was requested (40 to 90 minutes), but all of them reported dyspnea, headache, and burning of the eyes. Mental status seemed unaffected. During exposure to 10% CO_2 atmosphere the subjects reported extreme hyperventilation and dyspnea, restlessness and confusion. Progressive listlessness also occurred. For these reasons

the period of exposure was discontinued after 15-25 minutes. A study on cognitive performance (Sayers *et al*, 1987) conducted in 10 healthy subjects, compared the effects of a 20 minutes inhalation of different CO_2 concentrations (0- 4.5- 5.5- 6.5- 7.5)% and a 80 minutes inhalation of 6.5% CO_2. A greater impairment of cognitive performance was seen while subjects breathed 6.5% and 7.5% CO_2 compared to 0%, 4.5% and 5.5% CO_2. Exposure to 7.5% CO_2 caused marked discomfort in all cases and one subject withdrew before completing the tests. In another study (Maresh *et al*, 1997) on 32 healthy volunteers, 6% and 8% CO_2 inhalations were compared, and a feeling of derealization or depersonalisation was reported only in the 8% CO_2 condition. The subjects were able to perceive a difference between the two concentrations, although shortness of breath, sweating, heart palpitations, pressure in the chest, tingling, and dizziness occurred in both the conditions. Two subjects removed their mouthpiece and walked out from the experiment during the 8% CO_2 condition.

3. CO_2 challenge as an experimental model of panic attacks

The current interest in the use of CO_2 as a probe of experimental anxiety originates in 1984 by simultaneous observations that CO_2 triggers an immediate feeling of anxiety, which resembles naturally occurring panic, in subjects with a diagnosis of PD (Gorman *et al*, 1984; Van den Hout and Griez, 1984). Not only is the inhalation of CO_2 an efficient means of provoking panic and anxiety in individuals affected by PD, but it also is a relatively easy and non-invasive procedure, since the induced symptoms, while intense, are very short-lived. Based on this, CO_2-induced panic has become a subject of much research over the last decades and has contributed to the formulation of several influential theories on the aetiology of PD.

Different methodological approaches have been used to demonstrate that exposure to high concentration of CO_2 tends to produce intense bodily sensations and panic attacks, significantly more often in individuals with PD than in individuals with other disorders or in healthy controls. The two most common techniques of CO_2 administration are: 1) single or double vital capacity inhalation of 35% CO_2 through a self-administration mask (Verburg *et*

al, 2001); 2) 5-20 minutes inhalation of 5-7.5% CO_2 through continuous exposure to a steady-state level in a respiratory canopy, or through Read rebreathing method via mouthpiece (Papp *et al*, 1997). The assessments of the subjective response among the studies differ on whether they have used a self-report measure, a behavioural definition of panic, or an observation of the participant by the experimenter (Rassovsky and Kushner, 2003). While many instruments developed for this purpose tend to survey both physiological and psychological panic symptoms defined by the Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV-TR) (APA, 2000) criteria, there is wide variation in terms of the minimum symptom intensity threshold required to define a panic attack.

3.1 Validation of CO_2 as an experimental model of panic attacks

There is substantial variability among the studies in the rates of CO_2-induced panic attacks, both in individuals with PD and in controls. However, the pattern of the overall findings described below indicates that inhaling high doses of CO_2 is a validated method to induce panic.

After CO_2 inhalation PD patients experience panic attacks characterized by similar symptoms to those occurring in real life panic attacks (Gorman *et al*, 1984; Van den Hout *et al*, 1984). PD patients are more sensitive to CO_2 challenges than healthy controls (Fyer *et al*, 1987; Griez *et al*, 1990a; Griez *et al*, 1987b; Perna *et al*, 1994a; Woods *et al*, 1988; Woods *et al*, 1986). Furthermore, PD patients are more sensitive to CO_2 challenges than patients affected by other anxiety disorders, like Obsessive Compulsive Disorder (Griez *et al*, 1990a; Perna *et al*, 1995b; Zandbergen *et al*, 1991), General Anxiety Disorder (Holt and Andrews, 1989; Perna *et al*, 1999a; Verburg *et al*, 1995a), Specific and Situational Phobias (Rapee *et al*, 1992; Verburg *et al*, 1994), and other subjects with high level of anxiety (Gorman *et al*, 1988; Griez *et al*, 1990b). PD patients are also more sensitive to CO_2 challenges than patients affected by Major Depressive Disorder (Kent *et al*, 2001; Perna *et al*, 1995a; Verburg *et al*, 1998a) and Eating Disorders (Perna *et al*, 2004c). Specifically, PD patients with prominent respiratory symptoms appear to be more sensitive to CO_2 compared to PD patients with predominant non-respiratory symptoms (Biber and Alkin, 1999; Nardi *et al*, 2002; Valenca *et al*, 2002). Also, history of accidental suffocation and presence of premenstrual distress are

associated with a higher sensitivity to CO_2 inhalation (Monkul *et al*, 2010; Nillni *et al*, 2010).

Some exception to the specificity of CO_2 hyperreactivity has been observed: an intermediate sensitivity to CO_2 was observed in social phobia (Antony *et al*, 1997; Blechert *et al*, 2010; Caldirola *et al*, 1997; Gorman *et al*, 1990; Papp *et al*, 1993; Schutters *et al., 2012*), Premenstrual Mood Disorder (Gorman *et al*, 2001; Harrison *et al*, 1989; Kent *et al*, 2001) and Post-Traumatic Stress Disorder (Muhtz *et al*, 2011) but see (Talesnik *et al*, 2007), thus suggesting a possible shared underlying pathophysiology of those disorders with PD; moreover, preliminary findings suggest the presence of a certain vulnerability to CO_2 in Bipolar Disorder (MacKinnon *et al*, 2007; Mackinnon *et al*, 2009) and extreme sensitivity to CO_2 in Schizophrenic patients (Savitz *et al*, 2011).

3.2 Genetics of CO_2 sensitivity

A family history of PD conveys a liability to experience anxiety with CO_2 exposure. Studies on healthy individuals with family histories of PD suggest that a panic reaction to high-dose CO_2 is a trait marker for PD, in fact individuals with a family history of PD, but no personal history of panic attacks, respond to CO_2 inhalation with more anxiety than do individuals who lack a family history (Cavallini *et al*, 1999; Coryell, 1997; Coryell *et al*, 2001; Coryell *et al*, 2006; Perna *et al*, 1999b; Perna *et al*, 1995c; van Beek *et al*, 2000). Moreover, patients who panic with CO_2 inhalation appear to have a relatively familial form of the illness (Perna *et al*, 1996) and studies of twins from the general population have shown the response to be heritable (Battaglia *et al*, 2007; Battaglia *et al*, 2008; Bellodi *et al*, 1998). Furthermore, the genetic factors that lead to react intensively to a hypercapnic stimulus coincide at a considerable extent with those that influence liability to naturally occurring panic (Battaglia *et al*, 2007). Taken together, these findings suggest that CO_2 hypersensitivity runs in families as an endophenotype of liability to PD, albeit relatively independent of the full clinical manifestations of the syndromes of PD (Battaglia and Ogliari, 2005).

A polymorphism analysis across the exons of the lactate dehydrogenase (LDH) genes suggested that LDH polymorphisms might contribute to the variability to CO_2 respiratory challenge and discriminate well subjects at high risk for PD from low-risk control subjects.

Furthermore, the fear reaction to CO_2 administration appeared to be moderated by a polymorphism in the serotonin transporter (SERT) gene in healthy volunteers (Schmidt *et al*, 2000), however in PD patients no differences for measures of CO_2 reactivity among the SERT genotype groups were observed (Perna *et al*, 2004d). Recently, in a study from our laboratory using different dosages of CO_2, Schruers and coauthors confirmed that healthy individuals with the LL genotype of the SERT display a stronger fear reaction to CO_2, and showed this was particularly evident at intermediate CO_2 dosages (Schruers *et al*, 2011). Instead, at high dosages, or in particularly vulnerable individuals like in Perna's study, the administered stimulus was probably so powerful that a genetic effect causing increased vulnerability to CO_2 could no longer be detected.

3.3 Sensitivity to CO_2 in the normal population

As suggested by genetic studies (Coryell *et al*, 2001), subjective sensitivity to inhaled CO_2 seems to lay on a continuum distributed in the population. At one extreme of the CO_2 sensitivity continuum there are PD patients, especially those with prominent respiratory symptoms (Nardi *et al*, 2006a) and history of suffocation (Monkul *et al*, 2010). At the other extreme, there are patients affected by the majority of other disorders and healthy controls, especially without familial history of PD (see Table 1). A study by Battaglia and Perna (Battaglia and Perna, 1995) was aimed to set an ideal threshold of discrimination between patients and controls in terms of sensitivity to CO_2 challenges, by using receiver operating characteristic analysis of the responses of PD patients and healthy controls undergoing a single breath 35% CO_2 challenge. The increment of 26% of subjective anxiety proved to be the ideal threshold to separate PD patients from healthy controls.

Although weaker than in PD patients, however, a certain degree of CO_2 vulnerability can be evidenced in healthy individuals, if CO_2 dosage is sufficiently increased. Recent evidences have supported this view: a double-blind study by Bailey *et al.* (Bailey *et al*, 2005) on 20 healthy volunteers, with 20 minutes inhalation of 7.5% CO_2, showed significant increases, compared to placebo, in several affective dimensions, namely fear, anxiety, tension, sensation of threat, irritability, worry, feeling like leaving the room. The procedure significantly decreased feeling of being happy and relaxed. Poma and coauthors (Poma *et al*,

2005), with 7% CO_2 for 20 minutes, found similar results and further demonstrated a good test-retest reliability. In a single blind study on a small sample of healthy subjects (Kaye *et al*, 2004), showed a dose-response relationship with a single breath of 5, 25, and 35% CO_2 on subjective reports of anxiety, fear, breathlessness, difficulty in concentrating, and dizziness.

A study from our group (Griez *et al*, 2007) recently showed that CO_2 dose-dependently induced a condition complying with the formal criteria of panic in current psychiatric nosology. Sixty-four healthy subjects underwent a double inhalation of four mixtures containing respectively 0, 9, 17.5 and 35% CO_2, following a double blind, cross-over, and randomized design. Affective responses were assessed using an electronic visual analogue scale and a panic symptom questionnaire. The assessments were strictly based on the current nosologic criteria for panic. The healthy volunteers experienced a significant dose-dependent sense of "fear" or "discomfort" while reporting substantial panic symptomatology. Cognitive items from the symptom list as "derealization" and "fear of losing control" showed significant increases linked to the doses of inhaled CO_2. This finding demonstrates that CO_2 dose-dependently activates a condition identical to panic in healthy volunteers, regardless of any constitutional predisposition to psychiatric pathology. Moreover, older subjects displayed less behavioural vulnerability to CO_2, compared to younger individuals, consistent with the decline of natural PA's and the progressive blunting of panic symptomatology observed in PD patients when they grow older. Interestingly, two recent studies demonstrated higher vulnerability to CO_2 in females relative to male volunteers. These findings are in line with literature documenting sex-specific differences in panic psychopathology (Bunaciu *et al*, 2011; Nillni *et al*, 2012).

A factor structure analysis of the CO_2-induced panic symptoms in healthy volunteers (Colasanti *et al*, 2008) identified clusters of symptoms, respiratory, cognitive and neurovegetative, which were similar to those extracted in naturally-occurring panic in PD patients (Briggs *et al*, 1993; Meuret *et al*, 2006). This suggests a close similarity between natural panic attacks and the experimental CO_2 model in healthy volunteers. Interestingly, respiratory symptoms were found to be the strongest correlates of the subjective emotional response to CO_2 inhalation (Colasanti *et al*, 2008). Furthermore, it was found that a

multidimensional rating of CO_2-induced respiratory discomfort was able to discriminate between responders and non-responders to the CO_2 challenge, with a higher predictive power relative to that of all non-respiratory DSM-IV panic symptoms (unpublished data). The idea that the panic-like subjective internal state induced by CO_2 goes along with respiratory sensations fits clinical and epidemiological evidences that panic and respiration are closely linked (Goodwin and Pine, 2002; Nardi *et al*, 2009). Consistent with these evidences, a higher sensitivity to CO_2 inhalation is found in patients with respiratory symptoms and history of suffocation (Abrams *et al*, 2006; Biber *et al*, 1999; Monkul *et al*, 2010; Nardi *et al*, 2006a; Valenca *et al*, 2002). Furthermore, an interesting case report described a panic reaction to the inhalation of 35% CO_2 associated to dissociative symptoms and flashbacks in a healthy man with a history of near-drowning (Muhtz *et al*).

Recent studies investigated the effects of CO_2 on neurocognitive mechanisms underlying anxiety in healthy volunteers. In one study, the authors reported an experiment during which healthy participants underwent an emotional variant of the antisaccade test, a tool to assess the selective attention to environmental threats, during 20 minutes inhalations of both 7.5% CO_2 and normal air (Garner *et al*, 2011). CO_2 inhalation, compared to normal air inhalation, induced erroneous eye movements toward negative stimuli, suggesting that subjects were more readily orientated toward threat stimuli when exposed to CO_2 relative to normal air exposure. This effect was observed also in subjects who did not report subjective anxiety in response to CO_2, suggesting that the hypervigilance towards threat was independent of changes in subjective mood. In another study using a tracking task with a reaction time component and a complex target identification task in healthy volunteers, the inhalation of 7.5% CO_2 produced effects on task performance which were consistent with anxiety (Diaper *et al*, 2012).

Table 1) Continuum of sensitivity to single or double breath CO_2 challenges across the population.

Study Population	CO_2 challenge	CO_2–induced panic attack assessment	Panic rate	Ref.
16 PD Resp. subtype	35%CO_2 S.B.	4 PA s. + 1 cognitive s. + panic resembling real-life PA + agreement of 2 MD	93.7%	Valenca *et al*, 2002
28 PD Resp. subtype	35%CO_2 S.B.	Fear or panic + 4 PA s. + 1 cognitive s.	79%	Biber *et al*, 1999
23 PD Non-resp. subtype	35%CO_2 S.B.	Fear or panic + 4 PA s. + 1 cognitive s.	48%	Biber *et al*, 1999
32 PD	35%CO_2 S.B.	4 PA s. + 1 cognitive s.	46.9%	Monkul *et al*, 2010
11 FDR	35%CO_2 S.B.	Fear or panic + 4 PA s. + 1 cognitive s.	45.5%	Coryell, 1997
50 FDR	35%CO_2 S.B.	4 PA s. + increase in anxiety score>25/100	44%	van Beek *et al*, 2000
11 PD Non-resp. subtype	35%CO_2 S.B.	4 PA s. + 1 cognitive s. + panic resembling real-life PA + agreement of 2 MD	43.4%	Valenca *et al*, 2002
64 HV	35%CO_2 D.B.	4 PA s. + mean of the peak fear /discomfort score during placebo +2 SD	37%	Griez *et al*, 2007
32 FDR	35%CO_2 S.B.	4 PA s. + 1 cognitive s.	36.7%	Monkul *et al*, 2010
50 HV (low risk)	35%CO_2 S.B.	4 PA s. + increase in anxiety score>25/100	24%	van Beek *et al*, 2000
34 HV (low risk)	35%CO_2 S.B.	4 PA s. + 1 cognitive s.	23.5%	Monkul *et al*, 2010
23 FDR	35%CO_2 S.B.	Fear or panic + 4 PA s. + 1 cognitive s.	21.7%	Perna *et al*, 1995c
64 HV	17.5% CO_2 D.B.	4 PA s. + mean of the peak fear /discomfort score during placebo +2 SD	14%	Griez *et al*, 2007
64 HV	9% CO_2 D.B.	4 PA s. + mean of the peak fear /discomfort score during placebo +2 SD	6%	Griez *et al*, 2007
44 HV (low risk)	35% CO_2 S.B.	Fear or panic + 4 PA s. + 1 cognitive s.	2.3%	Perna *et al*, 1995c
15 HV (low risk)	35%CO_2 S.B.	Fear or panic + 4 PA s. + 1 cognitive s.	0%	Coryell, 1997

Table 1. The highest sensitivity to CO_2 challenges is observed in Panic Disorder patients with prominent respiratory symptoms. At the other extreme, there are healthy controls, especially without familial history of Panic Disorder. First Degree relatives of Panic Disorder patients display an intermediate sensitivity. Although weaker than in Panic Disorder patients and their first–degree relatives, a moderate degree of CO_2 vulnerability can be evidenced in healthy individuals, if CO_2 dosage is sufficiently increased (i.e. double breath 35% CO_2 challenge). PD: Panic Disorder; PA s.: panic attack symptoms; FDR: first-degree relatives of Panic Disorder patients; HV: healthy volunteers; low risk: no familiarity for Panic Disorder; S.B.: single breath; D.B.: double breath; MD: medical doctors; SD: standard deviation.

4 Pharmacological modulation of the affective response to CO_2

4.1 Studies in healthy volunteers

The serotonin (5-HT) system plays a modulatory role on CO_2-induced affective response: selective serotonin reuptake inhibitors (SSRIs) like paroxetine inhibit the response to 7.5% CO_2 (Bailey *et al*, 2007), while the administration of the 5-HT antagonist metergoline increased the anxiety provoked by CO_2 inhalation (Ben-Zion *et al*, 1999; Meiri *et al*, 2001). Studies using single breath 35% CO_2 showed that the manipulation of 5-HT precursors availability in healthy volunteers induced no clear effects on the response to single breath 35% CO_2 (Hood *et al*, 2006; Klaassen *et al*, 1998; Miller *et al*, 2000; Schruers *et al*, 2000a). A recent study from our laboratory (see Chapter 5) surprisingly showed that tryptophan depletion inhibited the response to a double-breath 35% CO_2 challenge and "tryptophan loading" (an amino-acid mixture containing 5.15 g l-tryptophan) tended to increase the vulnerability to CO_2. In a separate analysis, a significant positive relationship between plasma tryptophan and intensity of fearful response to CO_2 was found, indicating that a lower availability of 5-HT precursors is associated to a blunted affective response to CO_2. This is contrasting with the results of studies in PD patients (see below) showing that tryptophan depletion increased CO_2 sensitivity in PD patients while the administration of 5-hydroxtryptophan inhibited the response to CO_2 (Schruers *et al*, 2002b).

The pharmacological enhancement of GABA system, with both selective and non-selective benzodiazepine receptor agonists, results in inhibition of the response to CO_2 (Bailey *et al*, 2007; Bailey *et al*, 2009). Similarly, ethanol (another GABA enhancer) infusion also inhibited the response to CO_2 (Cosci *et al*, 2005). Other anxiolitics compounds like the β-blocker propranolol, the histamine receptor antagonist hydroxyzine or the D_2-antagonist flupentixol were not effective in reducing the CO_2-induced anxiety (Papadopoulos *et al*, 2010). The administration of cholecystokinin, an anxiogenic challenge per se, resulted in an inhibitory effect on the anxiety induced by CO_2 challenge (Pols *et al*, 1999; Schruers *et al*, 2000b). Biperiden (Battaglia *et al*, 2001), a muscarinic antagonist, appeared to protect against CO_2-induced anxiety while nicotine transcutaneus administration did not have any effects (Cosci *et al*, 2006).

The only two studies exploring the role of noradrenergic system in modulation of CO_2-response in healthy volunteers showed that premedication with the α_2-antagonist yohimbine (Pols *et al*, 1994) and the α_2-partial agonist clonidine (Woods *et al*, 1989) did not alter the response to CO_2. Preliminary indications suggest the novel anxiolytic CRF-antagonist compounds induce a decrease in CO_2-induced symptoms (Bailey *et al*, 2011). Finally, premedication with the opioid antagonist naltrexone did not have any effect on CO_2-vulnerability, which contrasts with the evidence of increased symptomatic response to a similar panicogenic challenge, lactate, after administration of naloxone (Preter *et al*, 2011).

4.2 Studies in Panic Disorder Patients

As a further confirmation of the validity of CO_2 as a model of panic, drugs that are effective in treating PD, like tryciclic antidepressants and SSRIs, also reduce the anxiety that patients with PD experience when they inhale CO_2. This has been shown for tricyclic antidepressants (Bertani *et al*, 1997; Gorman *et al*, 1997; Perna *et al*, 1997), benzodiazepines (Beckett *et al*, 1986; Nardi *et al*, 1999; Pols *et al*, 1996a; Pols *et al*, 1991; Roth *et al*, 1992; Sanderson *et al*, 1994; Woods *et al*, 1986; Woods *et al*, 1989), SSRIs (Bertani *et al*, 2001; Bertani *et al*, 1997; Perna *et al*, 2004a; Perna *et al*, 2002; Perna *et al*, 1997; Pols *et al*, 1996b), venlafaxine (Bertani *et al*, 2003) and the reversible monoamine-oxidase inhibitor toloxatone (Perna *et al*, 1994b). However, recently in a placebo-controlled study on 32 subjects who had either a past history of panic attacks or a family history of PD, or a panic reaction to CO_2 challenges, escitalopram did not produce greater changes than placebo in panic or anxiety responses after CO_2 exposure (Coryell and Rickels, 2009).

Other pharmacological challenges modulated the response to the challenge in PD patients: the 5-HT reuptake enhancer tianeptine decreased the vulnerability to CO_2 and tryptophan depletion increased CO_2 sensitivity in PD patients (Hood *et al*, 2006; Klaassen *et al*, 1998; Miller *et al*, 2000; Schruers *et al*, 2000a).

4.3 Studies in animal models

There is a relatively limited amount of studies exploring the use of CO_2 as a preclinical model of anxiety. It has been demonstrated that rats exposed to CO_2 showed increased levels of freezing relative to controls (Mongeluzi *et al*, 1996) and that the behavioral response to CO_2 was dose-dependent (Maren, 2009; Mongeluzi *et al*, 2003; Ziemann *et al*, 2009). The anxiogenic properties of CO_2 have also been shown using the open-field test, the aversion test, and context fear conditioning (Maren, 2009; Mongeluzi *et al*, 2003; Ziemann *et al*, 2009).

In a proconflict test in rats, exposure to 35% CO_2 enriched air significantly decreased the drinking behaviour associated to the delivery of response-dependent punishment, indicating increased anxiety. This effect was abolished with pre-treatment with alprazolam but not with flumazenil (Cuccheddu *et al*, 1995). Another study reported that CO_2 decreased the function of the GABA-A receptor complex in rat brain (Concas *et al*, 1993).

5. Putative mechanisms of CO_2-induced affective states

The neurobiological mechanisms underlying CO_2 anxiogenic properties still need to be elucidated, however in the last ten years a few studies have provided a number of very important clues which contributed to gain a better understanding of the relationship between CO_2 and affect: these clues are 1) the neuroimaging evidence of limbic and paralimbic activation during CO_2 inhalation; 2) the in-vitro demonstration of the existence of midbrain CO_2-chemosensitive serotonergic neurons, projecting to the brain regions involved in affect regulation; 3) the in-vitro/in-vivo evidence that amygdala is a chemosensitive region, which triggers fearful responses in response to a fall in pH, as in case of hypercapnic acidosis.

5.1 Neural response to CO_2 inhalation involves limbic and paralimbic activations

The neural activations during CO_2-inhalation have been studied with Positron Emission Tomography (PET) and $H_2[O_{15}]$ on nine healthy volunteers (Brannan *et al*, 2001; Parsons *et al*, 2001). When compared to the images acquired during inhalation of a N_2/O_2 gas mixture with the same apparatus, and also during paced breathing, and with eyes closed rest, inhalation of a gas mixture containing 8% CO_2 produced changes in cerebral blood flow in a number of brain structures associated with the control of motivational states and emotions: the network of activations included pons, midbrain (mesencephalic tegmentum, parabrachial nucleus, and periaqueductal gray), hypothalamus, limbic and paralimbic areas (amygdala and periamygdalar region), cingulate, parahippocampal and fusiform gyrus, anterior insula, caudate, pulvinar, and the cerebellum as well; strong deactivations were seen in prefrontal cortex and dorsal and posterior cingulated (Brannan *et al*, 2001; Parsons *et al*, 2001). Interestingly, the activations and deactivations were significantly correlated with the feeling of air hunger evoked by CO_2. Furthermore, the neural activations and deactivations were strikingly similar to those observed during other motivational/affective states involved in the regulation of homeostasis of the organism, such as pain, thirst, and hunger.

A recent study in PD patients using double breath 35% CO_2, with electroencephalogram (EEG) being recorded immediately after the inhalation, showed electroencephalographic changes associated to CO_2 – induced panic attacks, indicating a reduction in the frontal cortex activity, a lack of coherence between frontal and occipital areas, and an increased right posterior activity (Lopes *et al*, 2009).

Neuroimaging studies investigating the brain response to acute administration of CO_2 have been limited so far by technical and functional difficulties, and by the presence of artefacts such as head movements and large changes in blood flow produced by CO_2. An fMRI investigation of brain responses to CO_2 challenges in subjects with high and low CO_2–vulnerability is currently undergoing in our laboratory.

5.2 Midbrain serotonergic neurons are CO_2 chemosensitive

Converging evidences indicated the existence of midbrain serotonergic neurons acting as CO_2-chemosensors (see review (Corcoran *et al*, 2009)). A subset of 5-HT neurons is located in the so-called *chemosensitive* zone, the ventrolateral medulla. Serotonergic neurons have a crucial role in the detection of hypercapnic acidosis, as demonstrated by the fact all the raphe neurons stimulated by hypercapnia are serotonergic. Also, 5-HT neurons are associated with large arteries, a strategic position which allow them to faithfully sense CO_2 concentration (Bradley *et al*, 2002). In-vitro studies showed that 5-HT neurons increase their firing rate in response to CO_2 in rodents' brain and that the response is not dependent by inputs from other brain regions (Bradley *et al*, 2002; Severson *et al*, 2003). Studies in freely moving cats demonstrated that a subset of 5-HT raphe neurons increases their firing rate in response to hypercapnia, with some neurons displaying non-linear responses and a large increase in firing rates after response threshold (Veasey *et al*, 1995, 1997). This is supported by experiments using *in vivo* microdialysis showing that increased inhaled CO_2 causes a 2-3 folds increase in extracellular 5-HT levels (Kanamaru and Homma, 2007).

The functional role of these midbrain serotonergic neurons seems not just confined to control exclusively the ventilatory responses to CO_2, instead these neurons may be involved in the regulation of affective behaviors as well. On the basis of the demonstration of projections from midbrain serotonergic neurons to areas like forebrain and limbic system, Richerson proposed that these neurons also mediate non-ventilatory responses to hypercapnia, such as arousal, and feeling of anxiety and suffocation (Richerson, 2004). These observations are in line with the evidence from human studies indicating a modulatory role of the 5-HT system on the affective response to CO_2 (see paragraph 4).

5.3 Amygdala is a CO_2-chemosensor that elicits fear responses

A recent study by Ziemann *et al.* (Ziemann *et al*, 2009) provided a molecular explanation for how CO_2 elicits fearful responses. The work originated by the observation that the molecular channels permeable to Na^+ and Ca^{2+}, acid-sensing ion channel-1a (ASIC1a), are expressed on dendrites and neuronal bodies in the amygdala and are necessary for the expression of normal fearful responses in rodents. Using in-vitro and in-vivo techniques, including in-vivo

pH measurement during CO_2 inhalation, the authors showed that the inhalation of CO_2 reduced amygdalar pH, and that the reduction of pH led to the activation of amygdalar ASIC1a channels. Moreover, the study demonstrated that CO_2 inhalation elicited defensive behaviours such as freezing, increased aversion in the aversion test, reduced preference for open spaces in the open-field test, and context fear conditioning. Notably, the disruption of ASIC1a genes, or the acute inhibition of ASIC1a channels, blunted each of these responses.

These findings, all together, indicate not only that CO_2 directly triggers emotional responses by activating brain areas that control emotions, but also that CO_2 can be detected directly by structures involved both in control of respiration and emotions, such as midbrain and amygdala. These neurobiological evidences are in line with the previously reviewed findings from human studies, indicating that 1) acute inhalation of CO_2 induces an emotion strongly related to respiratory discomfort, which closely resemble natural-occurring panic (Colasanti *et al*, 2008), and that 2) exposure to CO_2 modulates the selective attention to threat in healthy individuals, independently of subjective changes in mood (Garner *et al*, 2011).

In the next section, we will discuss the idea, pointed out by the converging neurobiological evidences described above, that the response to CO_2 might represent an emotion serving an adaptive homeostatic function.

6. CO_2 as the agent of a primal emotion

6.1 Rostral extension of CO_2 sensitivity

Rising CO_2 lowers pH and increases ventilation; a fall in CO_2 increases pH and slows down ventilation. Thus, CO_2 levels regulate two basis elements of aerobic life, oxygen supply and acid base balance. This process is a matter of reflex adjustments mediated by chemosensitive neurons and autonomic centers in phylogenetically ancient brain structures of the rhombencephalon.

We now have evidence that CO_2 also drives specific neurons at higher levels in the central nervous system. Both the midbrain and the limbic system have proven to be CO_2 susceptible.

Midbrain dorsal raphe neurons are CO_2 sensors, which are not mainly, or not at all, involved in ventilatory control (Severson *et al*, 2003). These neurons project rostrally to evolutionarily more recent structures throughout the forebrain. They share 5-HT mediated CO_2 sensitivity with their medullary respiratory counterpart but apparently yield functional effects at the level of limbic structures.

Then comes the recent discovery that the amygdala itself, a keystone in the affective brain, directly senses CO_2 released acidity (Ziemann *et al*, 2009). Thus, CO_2 sensitivity extends into neuronal systems which, as far as we know, have no respiratory function. Once believed to be mainly a factor of reflex functions and the principal parameter of ventilatory control, CO_2 now appears as a potential player in higher functions in the brain.

6.2 Grounds for a suffocation alarm

Observing the behavioural vulnerability of PD subjects, Klein once proposed that panic attacks may be false biological alarms triggered by a suffocation detector that went oversensitive (Klein, 1993). The theory was met with scepticism, namely because ventilatory chemosensitivity proved normal in PD (Papp *et al*, 1995; Zandbergen *et al*, 1991). Yet the new evidence of CO_2 sensors higher up in the brain has changed the perspective.

Besides, CO_2 inhalation has been observed to induce dose dependently panic and anxiety-like affects in healthy individuals (Bailey *et al*, 2005; Griez *et al*, 2007; Schruers *et al*, 2011). This lends more credibility to the idea of an hardwired inborn, CO_2 driven alarm machinery in humans. Finally, it has been found that CO_2-provoked breathlessness caused the activation of limbic and paralimbic areas otherwise closely linked to emotional responses (Brannan *et al*, 2001; Evans *et al*, 2002; Liotti *et al*, 2001). Commenting on these findings, the authors note that the sense of suffocation evokes a strong emotion with a negative valence, and go on speculating that this may serve as an internal alarm in case of disrupted vital function (Liotti *et al*, 2001).

6.3 A particular instance of primal emotion

There is a generally accepted view that emotions have two components: sensation and intention. The former is the emergence of a disturbed bodily function into the stream of consciousness, "the mental representation of physiological changes" (Dolan, 2002). The latter is a move towards restoration of homeostasis. Primal emotions are those where the sensation component is interoceptor driven and linked to the basic functions of life and death such as water, food and air supply or thermal and acid base regulation (Denton, 2006).

In this latter perspective, panic might be considered as a perfect instance of primal emotion. The sense of neuronal CO_2 loading spreads rostrally throughout the brain and emerges into the conscious distress of panic. Biological distress permeates from the primitive neurovegetative rhombencephalon into the affective brain until the cingulate cortex and the painful awareness of a full-blown emotion. In this way, panic is the affective expression of suffocation, a scream for air and therefore a scream for life.

7. Conclusions

The experimental work and historical clinical observations reported in the present review demonstrated that the human organism has a behavioral vulnerability to increasing levels of acute hypercapnia. The subjective response to CO_2 observed in experimental studies has been expressed as a transient affective distress complying with the definition of panic in current psychiatric nosology.

PD patients and their relatives show a hypersensitivity to CO_2, which became considered a genetic marker of vulnerability to panic. However, experimental work in healthy volunteers and preclinical models clearly showed the panicogenic properties of CO_2 are not merely restricted to clinical populations.

The validity of the use of CO_2 in healthy volunteers as a reliable experimental model of panic would open interesting perspectives for its use in future research. Assuming that similar physiologic mechanisms underlie the response to CO_2 inhalation in at least a subset of

PD patients and in healthy volunteers, CO_2 challenges in healthy subjects may have a potential role in clinical research as an early test for novel compounds, and in experimental research as a tool for research on the pathophysiology of PD.

Finally, we have suggested the idea that the affective response to CO_2 and panic might represent an instance of "primal emotion", a concept proposed by other authors (Denton, 2006; Dolan, 2002) to describe those emotions driven by interoceptors, which are conceived to move the organism towards the restoration of internal homeostasis. Further research would be worth to see whether CO_2 in healthy subjects might indeed represent a valid empirical approach to study primal emotional systems in humans.

Conflict of interest

The authors declare no conflicts of interest.

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Chapter II

Carbon Dioxide inhalation induces dose-dependent and age-related negative affectivity

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Abstract

Background

Carbon dioxide inhalation is known to induce an emotion similar to spontaneous panic in Panic Disorder patients. The affective response to carbon dioxide in healthy subjects was not clearly characterized yet.

Methodology/Principal Findings

Sixty-four healthy subjects underwent a double inhalation of four mixtures containing respectively 0, 9, 17.5 and 35% CO_2 in compressed air, following a double blind, cross-over, randomized design. Affective responses were assessed according to DSM IV criteria for panic, using an Electronic Visual Analogue Scale and the Panic Symptom List. It was demonstrated that carbon dioxide challenges induced a dose dependent negative affect ($p<0.0001$). This affect was semantically identical to the DSM IV definition of panic. Older individuals were subjectively less sensitive to Carbon Dioxide ($p<0.05$).

Conclusions/Significance

CO_2 induced affectivity may lay on a continuum with pathological panic attacks. Consistent with earlier suggestions that panic is a false biological alarm, the affective response to CO_2 may be part of a protective system triggered by suffocation and acute metabolic distress.

Introduction

Intolerance to carbon dioxide in anxiety prone individuals has been widely documented (Coryell *et al*, 2006; Griez *et al*, 1998; Papp *et al*, 1997). When inhaling hypercapnic gasses, subjects diagnosed with Panic Attacks (PA) shortly sense an instant affect that closely replicates spontaneous panic (Coryell, 1997). Hence Klein inferred that pathological PA's may be false biological alarms, resulting from neuronal misfiring in an evolutionarily evolved, CO_2 driven oversensitive suffocation monitor (Klein, 1993).

In other words it was suggested that panic may be an inborn behavioural response to a metabolic distress. If so, panic must belong to the behavioural repertoire of healthy individuals, the hypersensitive alarm in PD subjects corresponding to a normoresponsive system in others. Accordingly, the very same mechanism firing false alarms in PD patients as a response to moderate CO_2 intake, should be activated in healthy subjects following higher doses of CO_2.

Here we demonstrate in healthy individuals that increasing concentrations of CO_2 dose dependently induce a negative affect and that this affect is semantically identical to panic, as defined in current psychiatric nosology.

Materials and methods

Subjects

Sixty-four volunteers provided their informed consent to participate in the study. There were 33 males and 31 females, aged 35.8 (SD=15.9) and 31.1 (SD=14.4) years respectively.

All potential participants had a complete inventory of medical history and a physical examination. Inclusion criteria were 18 to 65 years of age and a good present and past physical and mental condition. The mental condition was assessed by a structured psychiatric interview (Mini International Neuropsychiatric Interview) performed by a psychologist who was not directly involved in the study. Exclusion criteria included a history of pulmonary or

cardiovascular disease, the presence of hypertension (diastolic>100 mmHg; systolic>170 mmHg), cerebral aneurysm, pregnancy, epilepsy, excessive smoking (>15 cigarettes/day), use of adrenergic receptor blockers and use of psychotropic medication. A history of affective or anxiety disorders within a first-degree relative excluded participation. Participants were also excluded if they reported common specific fears or if there was any suspicion of history of Panic Attacks. The ethics committee of the Academic Hospital of Maastricht approved the study.

Procedure

The inhalation apparatus and the general procedures used in our laboratory have been described elsewhere (Verburg *et al*, 2001).

More specifically, the procedure consisted for each subject in a double inhalation of four mixtures containing respectively 0, 9, 17.5 and 35% CO_2 in compressed air, following a double blind randomized design.

Subjects were instructed in the use of a mask with a demand valve for self-administration of medical gasses and told that they would take a double vital capacity breath of four different concentrations of CO_2 in air, which, though being a harmless physiologic substance, may cause brief neurovegetative responses and arousal or anxiety, depending on the concentration. Subjects were asked to exhale to the maximum, to position the inhalation mask on their face and inhale their full capacity as quickly as possible. Next they were to empty their lungs and refill them immediately with gas, whereupon they had to hold their breath for 5 seconds before exhaling. All inhalations took place within one week, on four separate days but at the same time for each probant. Care was taken that each inhalation represented at least 80% of the subject's vital capacity.

Assessments

Affective responses were assessed with strict reference to the DSM IV (APA, 2000), which refers to a PA as "a discrete period of intense fear or discomfort", in which four (or more)

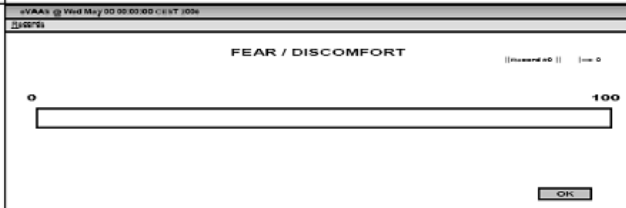
out of a list of thirteen predefined symptoms develop abruptly and reach a peak within 10 minutes.

Accordingly (Fig. S1) we used an electronic visual analogue scale for affect (eVAAS). The eVAAS was programmed on a Compaq Tablet PC, TC1000, with a 21 cm × 16 cm touch screen having a 1027 × 748 pixel resolution. The VAAS was a 20 cm × 1 cm horizontal bar. Subjects had to mark their anxiety level by tipping on the bar with a stylus, which had a 1 mm diameter spherical tip. The top of the display was labelled "*fear or discomfort".* The scale was anchored from 0, "no fear/discomfort at all", to 100, "the worst imaginable fear/discomfort". This instrument has been validated for use during 35% CO_2 challenges (van Duinen *et al*, 2008).

Panic Symptom List (PSL-IV) was used to evaluate panic symptomatology (Schruers *et al*, 2000a). It consisted in a questionnaire listing thirteen items, each item representing a DSM-IV panic symptom, to be rated on a five point scale, from 0 (absent) to 4 (very intense) (Fig. S1).

The eVAAS was presented at baseline immediately before inhalation, which was followed after CO_2 by multiple instant assessments, in fact as many as possible, during 60 seconds. This allowed the computation of both a peak value and an area under the curve (AUC). The Panic Symptom List was administered one minute before and after each inhalation. The total PSL score was calculated for each assessment.

Fig. S1) Experimental assessments. DSM IV criteria for Panic Attack; eVAAS for Fear/Discomfort; Panic Symptom List (PSL-IV)

DSM IV CRITERIA	EXPERIMENTAL ASSESSMENTS					
A discrete period of intense fear or discomfort in which four (or more) of the following symptoms developed…						
		NOT AT ALL	MILD	MODERATE	SEVERE	EXTREMELY SEVERE
1. Palpitations	PALPITATIONS, POUNDING HEART, OR ACCELERATED HEARTH RATE					
2. Sweating	SWEATING					
3. Trembling	TREMBLING OR SHAKING					
4. Sensation of shortness of breath or smothering	SENSATION OF SHORTNESS OF BREATH OR SMOTHERING					
5. Feeling of choking	FEELING OF CHOKING					
6. Chest Discomfort	CHEST PAIN OR DISCOMFORT					
7. Nausea or abdominal distress	NAUSEA OR ABDOMINAL DISTRESS					
8. Feeling dizzy, lightheaded or faint	FEELING DIZZY, UNSTEADY, LIGHTTHEADED, OR FAINT					
9. Feeling of derealization or depersonalization	DEREALIZATION (FEELING OF UNREALITY) OR DEPERSONALIZATION (BEING DETACHED FROM ONESELF)					
10. Fear of losing control	FEAR OF LOSING CONTROL OR GOING CRAZY					
11. Fear of dying	FEAR OF DYING					
12. Paresthesias	PARESTHESIAS (NUMBING OR TINGLING SENSATIONS)					
13. Chills or hot flushes	CHILLS OR HOT FLUSHES					

Statistical analysis

Statistical analysis was performed on the eVAAS peak values, obtained by subtracting the baseline from the maximum value, and PSL total scores, represented by delta scores (post-pre assessment).

A one-way Manova of repeated measures with eVAAS peak values as the dependent variable and dose (exposure to the four mixtures of CO_2) as the within-subjects factor was used to investigate the affective response to the various CO_2 mixtures. The same analysis was conducted with eVAAS AUC scores and PSL individual and total scores as the dependent variables.

AUC was calculated by the trapezoidal rule extrapolation method. The data were reanalysed using a repeated measures design with eVAAS peak values or PSL value as the dependent variable, age (below and above age 38) as a between-subjects factor and exposure to the four mixtures as the within-subjects factor. In both analyses, orthogonal polynomial trend contrasts were used to search for the presence of significant linear and/or quadratic trends in case of a significant "dose" effect.

Subjects were divided in "responders" and "non-responders", according to conservative criteria, proposed by others in previous CO_2 challenge studies (Poma *et al*, 2005). Following those criteria an arbitrary eVAAS peak score of 50 was used as threshold to identify the responders (mean of the eVAAS peak scored during AIR+2 SD). In addition responders should report at least one-point increase for at least four of the 13 PSL symptoms.

Results

Results are presented in figures 1-5. eVAAS peak values showed CO_2-triggered affectivity to be dose dependent ($p<0.001$), displaying an increase with concentration, which fits both a significant linear ($p<0.0001$) and quadratic pattern ($p<0.001$) (Fig. 1 a). eVAAS AUC values were also dose related ($p<0.0001$) (Fig. 2 a,b) and exhibited a significant linear and quadratic

pattern ($p<0.0001$). PSL data yielded similar results, with comparable dose-response relationship ($p<0.0001$) and mathematical pattern ($p<0.0001$) (Fig. 3 a).

Changes in individual PSL scores symptoms are presented in Fig. 4. As far as cognitive symptoms are concerned, a PSL score increase >1 in any of CO_2 conditions was shown in 53% of the subjects.

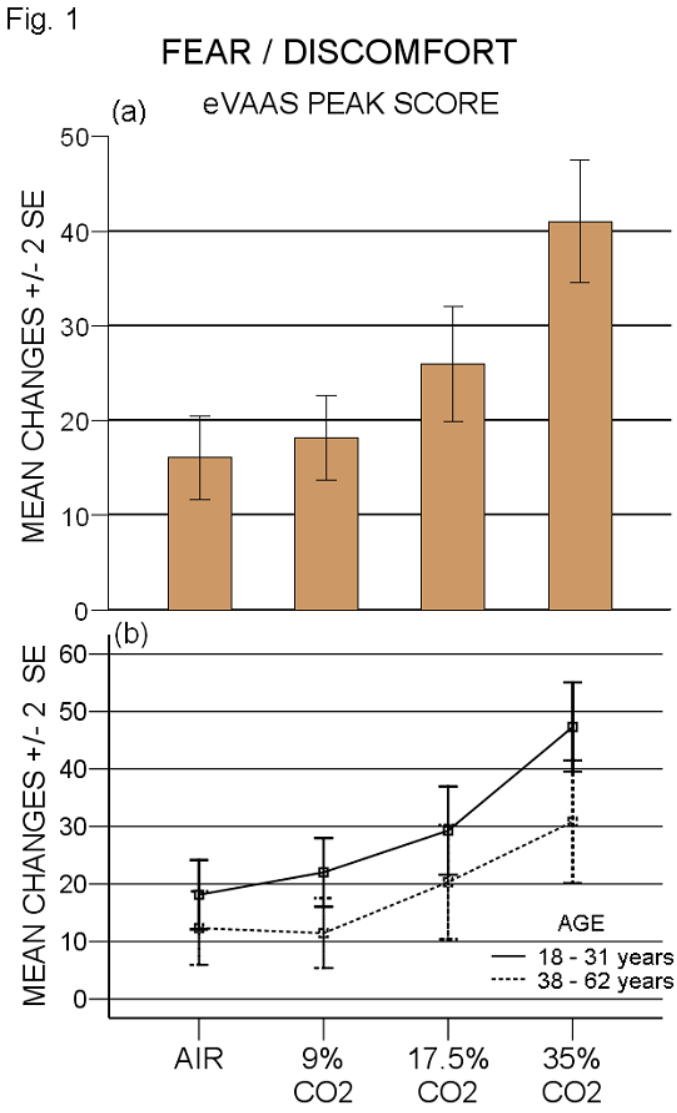
The sum of the symptom scores, defining a cognitive dimension ("derealization-depersonalization" score + "fear of loosing control-going crazy" score) (Cox *et al*, 1994; Meuret *et al*, 2006) is presented in Fig. 5. Looking to the cognitive symptoms separately (to which we added fear of dying), 48%, 26%, and 8% of the subjects reported an increase of PSL score respectively, in any of CO_2 condition. A significant CO_2 dose-dependent relationship was evidenced for derealization-depersonalization, and fear of losing control-going crazy ($p<0.0001$), which displayed a linear ($p<0.001$) and quadratic pattern ($p<0.05$). A similar significant dose-effect was found for fear of dying ($p<0.05$), however it appeared to be overall a very rare symptom.

According to above defined criteria there were 4 (6%), 4 (6%), 9 (14%), and 24 (37%) "responders" in the 0, 9, 17.5 and 35% CO_2 conditions respectively ($p\leq0.0001$).

Dividing the subjects in an older (>38 years) and younger group, analysis revealed a significant difference in eVAAS peak scores ($p<0.05$) (Fig. 1 b). eVAAS peak values in both age groups increase in a significant linear ($p<0.001$) and quadratic pattern ($p<0.001$) and run parallel. PSL scores displayed that age effect was not significant ($p=0.217$) (Fig. 3 b).

Dividing the subjects by gender, no significant differences were found between males and females in any of the assessed parameters (eVAAS peak score, eVAAS AUC score, PSL – IV score).

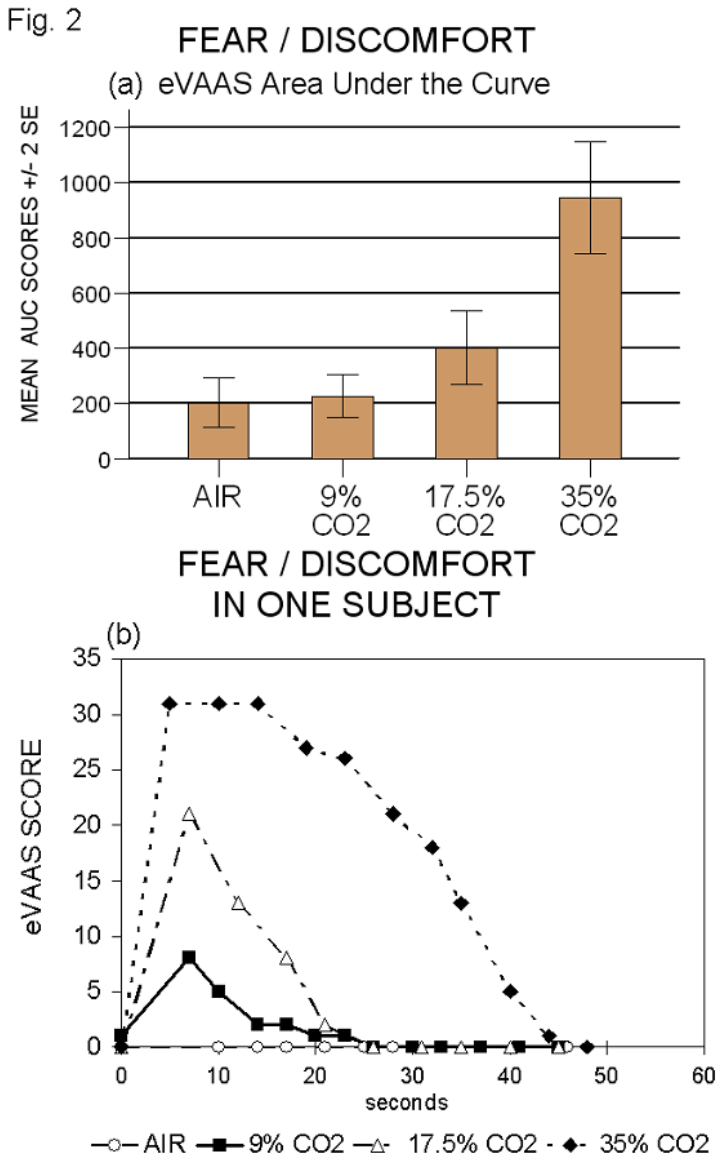
Fig. 1) Peak scores on the Fear / Discomfort scale in four different CO_2 conditions.



a) eVAAS: air vs 9% p=0.44; air vs 17.5% and vs 35% p≤0.001; 9% vs 17.5% and vs 35% p≤0.0001; 17.5% vs 35% p≤0.0001.

b) Younger versus older subjects: p<0.05

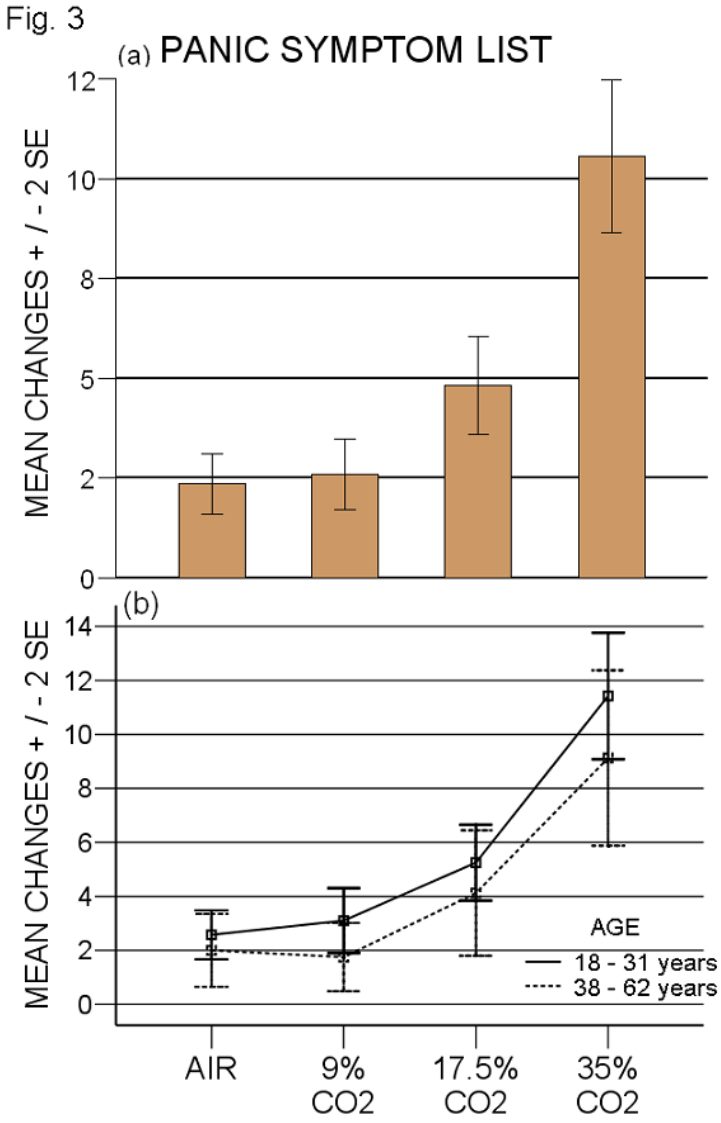
Fig. 2) Area under the curve on the Fear / Discomfort scale in four different CO_2 conditions.



a) eVAAS AUC score**:** air vs 9% p=0.91; air vs 17.5% and vs 35% p≤0.005; 9% vs 17.5% and vs 35% p≤0.001; 17.5% vs 35% p≤0.0001.

b) Time course of Fear/Discomfort in a single subject after the double inhalation of 0%, 9%, 17.5%, 35% CO_2 respectively.

Fig. 3) Intensity of PSL panic symptoms in four different CO_2 conditions.



a) PSL: air vs 9% p=0.76; air vs 17.5% and vs 35% p≤0.0001; 9% vs 17.5% and vs 35% p≤0.0001; 17.5% vs 35% p≤0.0001.

b) Younger versus older subjects: p=0.217.

Fig. 4) DSM panic symptoms intensity in healthy subjects taking four different doses of CO_2.

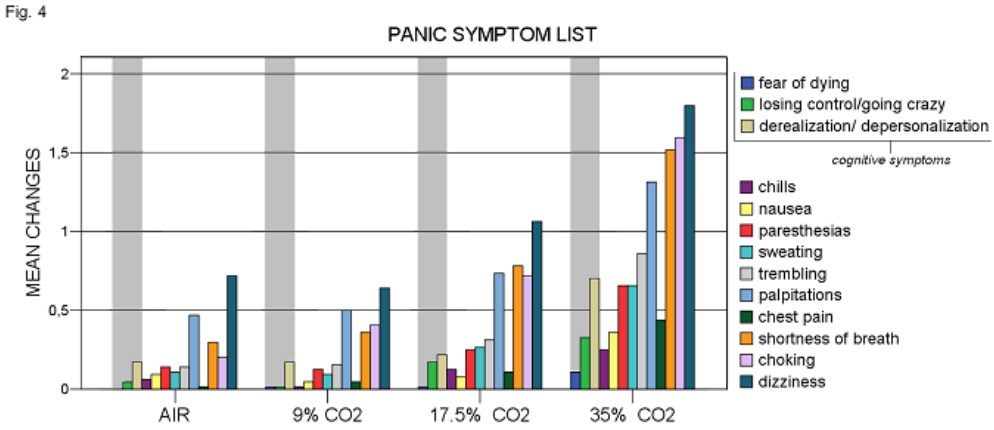
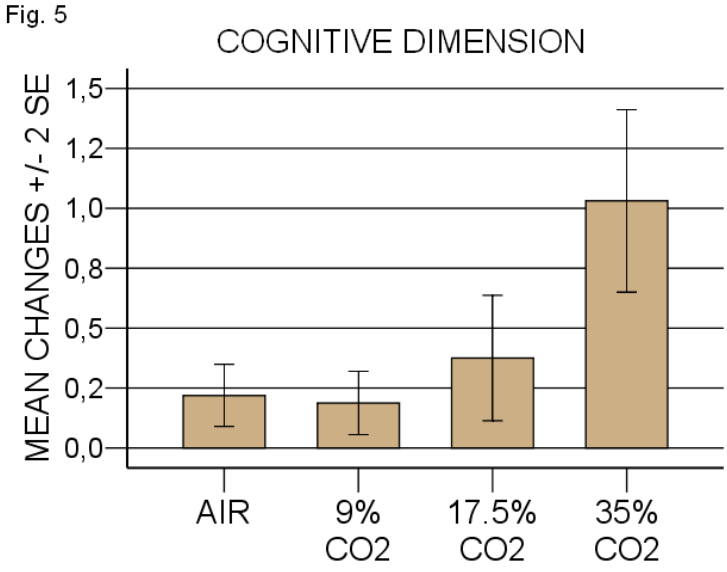


Fig. 5) Aggregate score of cognitive symptoms induced by four different CO_2 conditions in healthy subjects ("derealization-depersonalization" score + "fear of losing control-going crazy" score).



Discussion

The double breath challenge induced an instant affect with a negative valence. As rated on the eVAAS, the healthy volunteers experienced a significant sense of "fear" or "discomfort" while reporting substantial panic symptomatology on the PSL. Both eVAAS and PSL were strictly based on DSM-IV semantics. The picture as a whole was a mathematical function of CO_2 intake.

It may therefore be inferred that a double breath of increasing concentrations of CO_2 dose dependently induced a condition complying with the formal criteria of panic in current psychiatric nosology.

There has been a wealth of evidence showing that experimental hypercapnia triggers PA's in patients diagnosed with PD (Coryell, 1997; Griez *et al*, 1998; Papp *et al*, 1997), and conditions which are closely related to PD (Caldirola *et al*, 1997; Verburg *et al*, 1994). In contrast, the same procedure failed to affect patients with other disorders (Griez *et al*, 1990b), in particular those with Generalized Anxiety Disorder (Perna *et al*, 1999a; Verburg *et al*, 1995a), Obsessive-Compulsive Disorder (Griez *et al*, 1990a; Perna *et al*, 1995b), Eating Disorders (Perna *et al*, 2004c), Major Depression (Perna *et al*, 1995a) and control groups of healthy volunteers. First-degree relatives of PD patients however share a significant degree of CO_2 vulnerability (Coryell, 1997; Perna *et al*, 1995c; van Beek *et al*, 2000). In fact, the liability to experience panic with CO_2 exposure discriminates between individuals at high and low risk for PD (Coryell *et al*, 2006).

Recent reports have suggested that healthy individuals breathing a low 7% concentration of CO_2 may display signs of generalized anxiety (Bailey *et al*, 2005; Poma *et al*, 2005). Yet, the present results are the first to demonstrate that CO_2 dose dependently activates a condition identical to panic in healthy volunteers, regardless of any constitutional predisposition to psychiatric pathology.

Did CO_2 induce a true emotion? While formally meeting all the criteria of a PA according to modern psychiatric nosology, the CO_2 induced state we observed in our healthy subjects may have been a "phenocopy" of panic, the amalgam of autonomic symptoms of hypercapnia and some resulting physical discomfort. Amongst the 13 PSL items, we therefore separately analyzed the specific cognitive symptoms of "derealization" and "fear of losing control". Several studies have identified these symptoms as belonging to a specific psychological/cognitive cluster on basis of factor analysis (Cox *et al*, 1994; Meuret *et al*, 2006). Our results show that both "derealization" and "fear of losing control" were linked to the doses of inhaled carbon dioxide in a significant linear and quadratic pattern. Across the procedure, "derealization/depersonalisation" displayed more than one point increase (on a five point scale) in about half of the subjects, and "fear of losing control" in about one fourth of them. Fear of dying did not belong to the cognitive dimension in Meuret and Cox's studies (Cox *et al*, 1994; Meuret *et al*, 2006), nevertheless, from a conceptual point of view, it may refer to an extreme type of emotion. In the present study "fear of dying" remained very infrequent. However, when reported, we noted a significant dose-response relationship. It should be born in mind that all subjects were in a safe laboratory environment, and all had received ample reassurance regarding the safety of the intervention trough the informed consent procedure. This obviously influenced the psychological impact of CO_2. Yet, modest as they are, cognitive shifts did occur, they were a function of the experimental procedure, and their occurrence was statistically significant.

This lends support to the idea that, beyond a particular threshold, CO_2 may yield genuine psychotropic properties in healthy individuals.

Influential authors have increasingly referred to emotions as evolutionarily derived, "organism-ready solutions" to face major survival problems (Panksepp, 1998), as brain representations of internal body states (Damasio, 1999), and more specifically, as images of the "material me" arising from "the homeostatic condition of each individual's body" (Craig, 2003). The idea that panic may proceed from a suffocation alarm disrupted by acute CO_2 loading is perfectly consonant with such views. Several pieces of evidence point to a connection between hypercapnia and emotion. For instance, it appears that central chemosensitivity is not restricted to medullary respiratory neurons. Severson and co-workers

(Severson *et al*, 2003) have shown that midbrain raphe serotonergic neurons are CO_2 sensors, and midbrain neurons are not believed to have any direct function in the control of ventilation. Instead these midbrain chemosensors head mainly in the rostral direction. They have been proposed to participate in the homeostasis of the brain via non-respiratory responses to hypercapnia, including behavioural reactions as hyperarousal and anxiety (Richerson, 2004). Liotti and coauthors have produced neuroimaging evidence linking directly CO_2 inhalation with brain structures related to emotions (Liotti *et al*, 2001). Following CO_2 induced breathlessness, healthy volunteers displayed limbic and paralimbic activation, and neuronal firing in the affective brain correlated with the sense of suffocation. The authors comment that this neuronal activity may reflect a primal emotion, in other words "a compelling interoceptor-driven affect, rooted in metabolic distress, and aimed at signalling that the existence of the organism is endangered." In an earlier study on the characteristics of CO_2 induced responses, healthy volunteers spontaneously described their subjective experience as “frightening”, “panicky” or “scaring”, while authors noted that the sensitivity of the feeling, which was poorly correlated with the ventilatory response, varied threefold among individuals (Banzett *et al*, 1996).

Our study shows that CO_2 intake induces an affective state, which is similar to the psychiatric picture of panic. Within subjects, we observe a significant interaction between the intensity of the affective response and the CO_2 concentration of the inhaled mixture.

We show older subjects to display less behavioural vulnerability to CO_2, compared to younger individuals. To the extent that CO_2 intake is a valid model of panic, this difference between younger and older subjects strikingly evokes the decline of natural PA's and the progressive blunting of panic symptomatology in PD patients when they grow older (Sheikh *et al*, 2004). Most studies have found a lower prevalence of PD amongst elderly people (Krystal *et al*, 1992). The decreased CO_2 susceptibility in the elderly revealed by the present data reminds of a similar age effect found with experimental cholecystokinin provocation of panic (Flint *et al*, 1998). If midbrain serotonergic chemosensors are at work in the chain of events leading from CO_2 to panic, the phenomenon observed in our study might be related to an age dependent decline of serotonergic activity (Lawlor *et al*, 1989; Yamamoto *et al*, 2002).

No gender differences were found. This is somewhat surprising in view of all epidemiological data showing women to be at greater risk for panic than men (Gater *et al*, 1998). Yet, a recent study in a nonclinical population shows women reporting more fear and panic than men after CO_2 administration (Kelly *et al*, 2006). Interestingly, when asked to rate their experience on a "like or dislike" dimension (which dimension has a conceptual overlap with "discomfort"), the gender difference disappeared. This suggests women being more prone than men to report a feeling as “anxiety”. Therefore, lumping together anxiety and discomfort in our eVAAS may have blunted a gender effect. It is noteworthy that the few existing reports about sex differences in the so-called condition "non fearful PD", which diagnosis relies on “discomfort” rather than on “anxiety”, suggest that both genders have similar prevalence (Bringager *et al*, 2004; Fleet *et al*, 2000).

A final comment applies to the potential of further work with CO_2 challenges in healthy individuals. The panic model of CO_2, in particular the single breath 35% CO_2 procedure, has proven to be both valid and reliable (Verburg *et al*, 1998b). It has undergone extended pharmacological validation (Bertani *et al*, 1997). Assuming that higher doses of CO_2 activate the same physiologic chain of events in panic free individuals, CO_2 challenges in healthy volunteers may have a strong potential as a substitute to early clinical trials in testing novel pharmacological compounds.

In conclusion, it appears that healthy individuals display a distinct behavioural vulnerability to increasing levels of acute hypercapnia. This effect is dose-dependent and shares a striking similarity with the psychiatric condition of panic.

CO_2 susceptibility, sensed as acute affective distress may represent an evolutionarily evolved protective mechanism in case of impending asphyxia.

Chapter III

Carbon Dioxide-induced emotion and respiratory symptoms in healthy volunteers

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Abstract

A number of evidences have established that panic and respiration are closely related. Clinical studies indicated that respiratory sensations constitute a discrete cluster of panic symptoms and play a major role in the pathophysiology of panic. The aim of the present study was to explore the phenomenology of an experimental model of panic in healthy volunteers based on the hypothesis that: (1) we can isolate discrete clusters of panic symptoms, (2) respiratory symptoms represent a distinct cluster of panic symptoms, and (3) respiratory symptoms are the best predictor of the subjective feeling of panic, as defined in the DSM IV criteria.

Sixty-four healthy volunteers received a double inhalation of four mixtures containing 0, 9, 17.5, and 35% CO_2, respectively, in a double-blind, cross-over, random design. An electronic visual analogue scale and the Panic Symptom List (PSL) were used to assess subjective "fear/discomfort" and panic symptoms, respectively. Statistical analyses consisted of Spearman's correlations, a principal component factor analysis of the 13 PSL symptoms, and linear regressions analyses.

The factor analysis extracted three clusters of panic symptoms: respiratory, cognitive, and neurovegetative (r^2=0.65). Respiratory symptoms were highly related to subjective feeling of fear/discomfort specifically in the CO_2-enriched condition. Moreover, the respiratory component was the most important predictor of the subjective feeling of "fear/discomfort" (β=0.54).

The discrete clusters of symptoms observed in this study were similar to those elicited in panic attacks naturally occurring in patients affected by Panic Disorder. Consistent with the idea that respiration plays a crucial role in the pathophysiology of panic, we found that respiratory symptoms were the best predictors the subjective state defined in the DSM IV criteria for panic.

Introduction

Panic attacks (PAs) are defined in the current nosology as discrete periods of intense fear or discomfort accompanied by diverse physical and cognitive symptoms (APA, 2000). The symptomatology includes cardio-respiratory symptoms (dyspnea or breathlessness, palpitations, and chest pain); neurovegetative symptoms (nausea and sweating), and other nonspecific symptoms (trembling, paresthesias, dizziness, and chills). Frequent cognitive symptoms include feelings of derealization or depersonalization, fear of losing control or going crazy, and fear of dying.

Research from the past two decades has focused considerable attention on elucidating the phenomenology of panic, resulting in the recognition that PAs are characterized by inter-individual heterogeneity. Researchers have identified distinct subtypes of Panic Disorder (PD) on the basis of a predominant symptom constellation (Aronson and Logue, 1988; Bass *et al*, 1987; Ley, 1992). Such an approach may contribute to a better understanding of the etiology of panic since dysregulation in different psychobiological mechanisms may underlie phenotypically similar attacks. Gorman and coworkers in 1999 posed a neuroanatomical hypothesis of PD based on the idea that the physiological and behavioral consequences of a panic attack are mediated by a "central fear network" in the brain that is centred in the amygdala and involves its interaction with the hippocampus, medial prefrontal cortex, and hypothalamic and brainstem sites (Gorman *et al*, 2000).

Even though clustering of panic symptoms has not yielded always consistent results, there is general agreement about the existence of a prominent respiratory subtype of panic that is characterized by the prevalence of respiratory symptoms. Briggs and coauthors (1993) analyzed a sample of 1168 PD patients and divided them into two clusters. The first cluster, labelled “respiratory”, was characterized by the presence of shortness of breath, choking, fear of dying, chest pain, and tingling; the second “nonrespiratory” cluster was characterized by the absence of respiratory symptoms and the presence of the other symptoms listed in DSM-IV criteria for panic attack. Consistently with Briggs, Argyle and Roth (1989) and De Beurs *et al.* (1994) found a similar “respiratory” cluster in their factor analysis. Cox *et al.* (1994) extracted three factors: a dizziness-related symptom cluster, a cardio-respiratory distress

symptom cluster, and a catastrophic cognition symptom cluster. The cardio-respiratory cluster was primarily characterized by palpitations but also included dyspnea, choking, and fear of dying. Recently, Meuret *et al.* (2006) analyzed 343 PD patients and extracted three "dimensions" interpreted as cardio-respiratory, mixed somatic-autonomic, and cognitive. The cardio-respiratory cluster included shortness of breath, chest pain, fear of dying, palpitations, tingling, and choking. Shioiri *et al.* (1996), in a Japanese study of 207 PD patients, found slightly different results. Their cluster analysis revealed three panic symptom clusters. The first was comprised exclusively of physiological symptoms, among which respiratory symptoms were prominent. The second included mixed panic symptoms, including agoraphobia and anticipatory anxiety, and the third was comprised of fearful cognitive symptoms plus paresthesias and chest pain.

The existence of a distinct "prominent respiratory" subtype of panic seems to be supported by clinical evidence. Briggs *et al.* (1993) demonstrated that patients with the respiratory subtype of panic suffer more spontaneous PAs than patients with the residual subtype and that they respond better to imipramine than to alprazolam. The respiratory subtype has been shown to be associated with increased familial risk of PD (Horwath *et al*, 1997; Nardi *et al*, 2003; Nardi *et al*, 2006a; Nardi *et al*, 2006b), to be more common among heavy smokers, to have a longer duration and major severity of illness (Biber and Alkin, 1999), and to have a lower resting end-tidal pCO_2 (Moynihan and Gevirtz, 2001) and a higher sensitivity to CO_2 inhalation (Abrams *et al*, 2006; Biber and Alkin, 1999; Nardi *et al*, 2006a; Nardi *et al*, 2006b; Valenca *et al*, 2002). Other clinical features displayed by PD patients with the respiratory subtype include a later onset of the disease and a faster response to nortriptyline (Nardi *et al*, 2003; Nardi *et al*, 2006a; Nardi *et al*, 2006b), more past traumatic suffocation experiences and respiratory diseases (Bouwer and Stein, 1997; Verburg *et al*, 1995b), higher levels of anxiety sensitivity, and more panic-agoraphobic spectrum symptoms (Onur *et al*, 2007).

A strong relation between panic and respiration would be consistent with the high co-morbidity of respiratory disorders (asthma, COPD) and PD observed in large, epidemiological studies (Goodwin and Pine, 2002). Taken together, the data emerging from clinical, experimental, and epidemiological studies on panic and respiration suggest that the

neurobiological mechanism underlying PD may involve respiratory control (Battaglia *et al*, 2005; Klein, 1993; Preter *et al*, 2008). Briggs, Meuret, Biber, Nardi and co-workers have suggested that: (1) panic symptoms can be grouped into clusters, (2) respiratory symptoms constitute a distinct and well-defined cluster, and (3) respiratory symptoms play a major role in its pathophysiology.

In the present study, we sought evidence to support these suggestions by analyzing an experimental model of panic. We recently showed that increasing concentrations of CO_2 dose dependently induce a negative affect, which eventually culminates in genuine panic, also in healthy individuals (Griez et al, 2007). Accordingly, high doses of CO_2 may be considered as a model of panic in healthy subjects, just as moderate doses are in PD patients. On these premises, we performed a statistical analysis of the symptom pattern observed in our 2007 dose response experiment. In line with the above mentioned authors, we hypothesized that: (1) there are discrete clusters of panic symptoms, (2) respiratory symptoms can be extracted as an independent component, and (3) respiratory symptoms are, indeed, the best predictor of the subjective disturbance defined in the DSM IV criteria for panic.

Materials and methods

Subjects

Students and staff members were recruited by word of mouth from within Vijverdal Psychiatric Hospital in Maastricht and from the University of Maastricht. They received course credit for their participation in the studies. All potential participants provided a complete medical history and underwent a physical examination. Inclusion criteria were age 18-65 years and a good present and past physical and mental condition, based on the results of a structured psychiatric interview (Mini International Neuropsychiatric Interview) performed by a psychologist not directly involved in the study. Exclusion criteria included a history of pulmonary or cardiovascular disease, the presence of hypertension (diastolic>100 mmHg; systolic>170 mmHg), cerebral aneurysm, pregnancy, epilepsy, excessive smoking (>15 cigarettes/day), use of adrenergic receptor blockers, and use of psychotropic

medication. Individuals with a history of affective or anxiety disorders in a first-degree relative were excluded. Individuals with a present or past history of substance-related disorders, including caffeine-related disorder, were excluded. Participants were also excluded if they reported common specific fears or if they had a history of PAs. The ethics committee of the University of Maastricht approved the study. A complete description of the study was given both orally and in written form to the subjects. Those who met the inclusion criteria and who provided written informed consent were included in the study.

Procedure

The study was conducted according to a double-blind, randomized, cross-over design. All subjects received all of the following challenges: air: 0% CO_2, 20% 0_2, 80% N_2; low: 9% CO_2, 20% 0_2, 71% N_2; medium: 17.5% CO_2, 20% 0_2, 62.5% N_2; high: 35% CO_2, 20% 0_2, 55% N_2 in randomized order. Each subject underwent the four different challenges on four different days within the same week, at the same hour, to eliminate circadian effects in the challenge response. Every challenge was performed in the morning, between 9:00 and 12:00. The participants were asked to refrain from drinking beverages containing xanthine for at least 8 hours and from eating for at least 2 hours before the test.

The procedure was standardized for all subjects and for all sessions and was performed according to the protocol for 35% CO_2 inhalation used at the Maastricht Academic Anxiety Center (Griez *et al*, 1987a). The different gas mixtures were all delivered through the same nasal-oral exercise self-administration face mask using a double vital capacity inhalation technique. Before the challenge was started, the inspired vital capacity of every subject was evaluated using an analogue respirometer (Wright respirometer Mark 20) connected to the self-administration mask. The same respirometer measured the gas volume delivered at each inhalation. Baseline inspired vital capacity with a double breath of normal air was measured on each occasion, and a test breath was considered adequate if it was more than 80% of the baseline vital capacity. The subjects were then given the self-administration mask and told to hold it in their hands and to exhale as deeply as they could. A double-breath inhalation of a

mixture containing one of the four different concentrations of CO_2 was then delivered to the subjects. They were asked to take a maximal inspiration through the self-administration mouthpiece and to make a complete expiration outside the mouthpiece, immediately followed by a second maximal inspiration through the mouthpiece following the same procedure. At the end of the second inhalation, the subjects were asked to hold their breath for 4 seconds to enhance the alveolar gas exchange.

Measures

Assessments were chosen with strict reference to the definition of PA in the current psychiatric nosology, and specifically the DSM IV TR criteria for PA. These define a PA as a discrete period of intense fear or discomfort followed by the abrupt development of 13 well-defined symptoms. An electronic visual analogue scale for affect (eVAAS) was developed to record the subjective feeling of fear or discomfort. It consists of a Compaq laptop touch screen personal computer interfaced with original eVAAS software, in which the analogue scale is shown on the electronic display and the subject interacts directly via an electronic pen with the touch screen. The top of the display is labelled "fear or discomfort". The participants were instructed to indicate the amount of the subjective disturbance, in case of feeling either fear or discomfort. The visual analogue scale is represented by a 200-mm bar on the display that ranges from 0 (no fear/discomfort at all) to 100 (the worst imaginable fear/discomfort). This instrument has been validated for use during 35% CO_2 challenges (van Duinen *et al*, 2008).

Before each challenge began, the participants rated their baseline level of fear/discomfort. Panic symptoms were evaluated using the Panic Symptom List (PSL-IV) (Schruers *et al*, 2000a). This consists of a questionnaire listing 13 items, each representing one of the DSM-IV TR symptoms (i.e., palpitations; sweating; trembling; sensations of shortness of breath or smothering; feeling of choking; chest discomfort; nausea or abdominal distress; feeling dizzy, lightheaded, or faint; derealization or depersonalization; fear of losing control; fear of dying; paresthesias; chills or hot flushes). The participants were asked to rate the intensity of each symptom from 0 (absent) to 4 (very intense). The total scores thus ranged from 0 to 52.

Baseline assessment of eVAAS and PSL-IV symptoms were obtained one minute before each double inhalation (baseline score). One minute after the end of each double inhalation, the subjects filled in both the eVAAS and the PSL-IV questionnaire. At that time, they were asked to indicate the worst moment they experienced after inhaling the gas mixture (post-inhalation score).

The post-inhalation change was calculated by subtracting the baseline score from the post-inhalation score.

Statistical analysis

The statistical analyses were performed using SPSS 13.0. First, an ANOVA for repeated measures was conducted on the eVAAS score, total PSL score, and each of PSL symptom scores. The subsequent analysis of the relationship between eVAAS score and PSL symptoms consisted of four steps:

1) Spearman's non-parametric correlation analysis between eVAAS score and each of the 13 PSL-IV symptoms;

2) First linear regression analysis to determine the relative weight of each PSL-IV symptom in the determination of the eVAAS score. The dependent variable was the eVAAS score and the predicting variables were the PSL-IV symptoms;

3) Principal component factor analysis with varimax rotation to obtain variables with very high factor weights in some factors and very low factor weights in others. To insert a variable into a factor, a factor weight of at least 0.5 was selected as the threshold value. Factor scores were then computed as variables, representing a measure of each subject's contribution to each factor.

4) Second linear regression analysis to determine the relative weight of each principal component in the determination of the eVAAS score. The dependent variable was the eVAAS score and the predicting variables were the principal factor scores.

The correlation analysis and first linear regression analysis were made separately for each condition (air, low, medium, high). The principal component factor analysis was applied to the 13 PSL-IV symptoms only in the high condition (35% CO_2). The second linear regression analysis was performed only in the high condition.

Results

Sixty-four volunteers were included in the study: 33 males and 31 females aged 35.8 (15.9 SD) and 31.1 (14.4 SD) years, respectively.

PSL-IV scores for each symptom are presented in Table 1. Dizziness, sensation of shortness of breath, feeling of choking, and palpitations had the highest mean scores in all three CO_2 conditions (high, medium, and low). For both the eVAAS score and the PSL-IV total score, a statistically significant dose-response relationship was observed (these results are extensively presented elsewhere) (Griez *et al*, 2007). Each PSL symptom score was significantly dose-dependent ($p<0.05$).

Table 1) Panic Symptom List scores.

DOSE	AIR		LOW		MEDIUM		HIGH	
	mean	SD	**mean**	SD	**mean**	SD	**mean**	SD
Palpitations	**0,47**	0,73	**0,50**	0,70	**0,69**	0,76	**1,31**	1,06
Sweating	**0,00**	0,44	**0,10**	0,56	**0,25**	0,54	**0,63**	0,81
Trembling	**0,14**	0,43	**0,16**	0,52	**0,30**	0,69	**0,81**	0,97
Shortness of breath	**0,30**	0,55	**0,37**	0,73	**0,72**	0,82	**1,47**	1,16
Feeling of choking	**0,20**	0,44	**0,40**	0,73	**0,67**	0,79	**1,56**	1,13
Chest pain	**0,02**	0,22	**0,05**	0,28	**0,07**	0,31	**0,39**	0,71
Nausea	**0,09**	0,50	**0,05**	0,28	**0,08**	0,33	**0,29**	0,82
Dizziness	**0,72**	0,92	**0,63**	0,71	**1,03**	0,84	**1,74**	1,13
Derealization	**0,17**	0,38	**0,18**	0,43	**0,21**	0,58	**0,66**	0,90
Fear of losing control	**0,05**	0,21	**0,02**	0,22	**0,16**	0,49	**0,29**	0,66
Fear of dying	**0,00**	0,00	**0,02**	0,13	**0,02**	0,13	**0,10**	0,39
Paresthesias	**0,14**	0,39	**0,11**	0,32	**0,20**	0,48	**0,65**	1,03
Chills	**0,06**	0,24	**0,02**	0,34	**0,08**	0,33	**0,19**	0,54
PSL tot	**2,47**	3,07	**2,60**	3,51	**4,48**	4,01	**10,08**	7,07
E-VAAS	**16,06**	17,98	**17,66**	18,56	**26,16**	24,19	**44,94**	26,62

DSM panic symptoms intensity (mean ±SD) in four different CO_2 conditions.

Correlations

Correlation coefficients are shown in Table 2. A high Spearman's rho coefficient was found between the symptoms "feeling of choking" and "sensation of shortness of breath" in all conditions except air. The symptoms "derealization or depersonalization", "fear of losing control", and "fear of dying" were highly related to each other in all of the conditions.

Correlation coefficients between eVAAS score and PSL-IV scores across the four different doses are presented in Fig. 1. Dizziness and palpitations were strongly related to the eVAAS score in all of the CO_2 conditions, including the air condition. Sensation of shortness of breath and feeling of choking were strongly related to the eVAAS score in all of the CO_2 conditions but not in the air condition.

Regression analysis (1)

A regression analysis was performed to show the weight of each symptom in predicting the eVAAS score. Figure 2 shows the relative weight (β score) of each PSL-IV symptom separately for the four conditions. In the high condition, "feeling of choking" had the highest Beta score. "Sensation of shortness of breath" appeared to be a poor predictor. Looking at the other conditions, it is evident that the weight of "feeling of choking" in predicting the eVAAS score increased with the dose, while the weight of "shortness of breath" decreased with the dose. In the air condition, both respiratory symptoms had a low predicting value (<0) compared to "palpitations", "chest pain", and "dizziness", which had Beta scores higher than 0.3.

Table 2) Correlations between Panic Symptom List scores.

HIGH

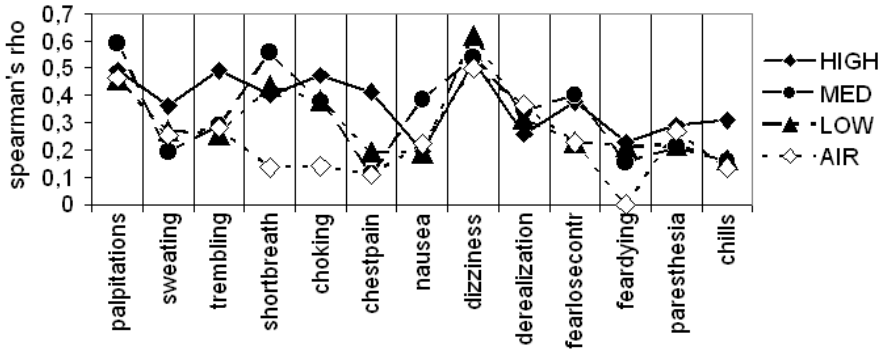
		VAS	palpitations	sweating	trembling	shortbreath	choking	chestpain	nausea	dizziness	derealization	fearlosecontr	feardying	paresthesia	chills
MED	VAS	1,00	0,49	0,36	0,49	0,40	0,47	0,41	0,19	0,52	0,26	0,38	0,23	0,29	0,31
	palpitations	0,59	1,00	0,51	0,51	0,40	0,29	0,44	0,18	0,43	0,26	0,37	0,11	0,25	0,22
	sweating	0,19	0,10	1,00	0,43	0,47	0,36	0,34	0,16	0,35	0,24	0,39	0,35	0,23	0,33
	trembling	0,29	0,17	0,32	1,00	0,19	0,20	0,54	0,31	0,52	0,30	0,27	0,20	0,43	0,41
	shortbreath	0,56	0,44	0,06	0,19	1,00	0,76	0,42	0,09	0,46	0,28	0,34	0,30	0,27	0,19
	choking	0,38	0,36	0,14	0,21	0,48	1,00	0,39	0,10	0,45	0,26	0,23	0,23	0,16	0,22
	chestpain	0,11	0,32	0,14	0,01	0,22	0,36	1,00	0,30	0,52	0,42	0,27	0,22	0,51	0,45
	nausea	0,39	0,28	0,16	0,28	0,35	0,18	0,11	1,00	0,26	0,34	0,38	0,30	0,22	0,46
	dizziness	0,54	0,49	0,06	0,13	0,38	0,20	0,26	0,31	1,00	0,27	0,24	0,26	0,33	0,24
	derealization	0,34	0,19	0,23	0,06	0,41	0,30	0,15	0,40	0,41	1,00	0,51	0,47	0,42	0,31
	fearlosecontr	0,40	0,30	0,04	0,23	0,56	0,41	0,19	0,51	0,41	0,73	1,00	0,60	0,25	0,28
	feardying	0,15	0,08	-0,07	-0,07	0,22	0,23	-0,04	-0,03	0,16	0,33	0,36	1,00	0,34	0,33
	paresthesia	0,21	0,11	0,40	0,19	0,16	-0,06	0,05	0,23	0,28	0,26	0,23	-0,05	1,00	0,37
	chills	0,16	0,13	0,32	0,03	0,35	0,23	0,28	0,15	0,11	0,20	0,25	-0,04	0,35	1,00
	VAS	1,00	0,46	0,27	0,26	0,43	0,38	0,19	0,19	0,62	0,32	0,23	0,21	0,22	0,16
	palpitations	0,46	1,00	0,25	0,32	0,13	0,12	0,16	0,21	0,26	0,18	0,19	0,12	0,06	0,17
	sweating	0,25	0,26	1,00	0,34	0,24	0,13	0,40	0,11	0,21	0,34	0,19	-0,04	0,29	0,45
	trembling	0,28	0,32	0,26	1,00	0,28	0,23	0,22	0,22	0,35	0,30	0,15	-0,04	0,37	-0,03
	shortbreath	0,13	0,32	0,11	0,10	1,00	0,61	0,40	0,17	0,24	0,29	0,35	0,26	0,16	0,00
	choking	0,14	0,36	0,06	0,02	0,28	1,00	0,20	0,16	0,33	0,17	0,35	0,26	0,15	0,09
	chestpain	0,11	0,25	0,15	0,15	-0,04	0,15	1,00	0,18	0,08	0,41	0,25	-0,02	0,49	-0,01
	nausea	0,22	0,11	0,49	0,21	0,02	0,21	0,24	1,00	0,18	0,26	-0,01	-0,02	0,31	0,21
	dizziness	0,50	0,40	0,26	0,22	0,17	0,31	0,10	0,25	1,00	0,44	0,20	0,21	0,34	0,11
	derealization	0,36	0,32	0,27	0,24	0,15	0,22	-0,22	0,22	0,44	1,00	0,20	0,29	0,55	0,14
	fearlosecontr	0,23	0,27	0,29	0,28	0,08	0,26	-0,02	-0,04	0,29	0,49	1,00	0,58	-0,02	0,29
	feardying	.	.	.	.	.	.	.	.	.	.	.	1,00	-0,04	-0,02
	paresthesia	0,27	0,24	0,12	0,20	0,33	0,33	0,19	0,26	0,30	0,21	-0,09	-0,04	1,00	0,16
	chills	0,13	0,19	0,08	-0,09	0,13	0,23	-0,02	0,18	0,17	0,22	-0,06	-0,02	0,31	1,00

AIR

LOW

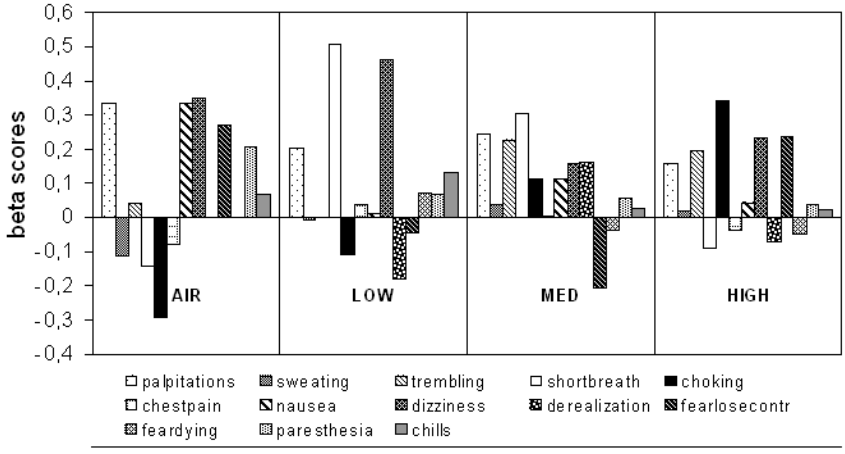
Spearman's correlation coefficients between DSM panic symptoms in four different CO_2 conditions.

Fig. 1) Correlations between Panic Symptom List scores and eVAAS Fear/Discomfort scores.



Spearman's correlation coefficients between DSM panic symptoms and Fear/Discomfort scores in four different CO_2 conditions. Sensation of shortness of breath and feeling of choking show a high correlation coefficient in all of the CO_2 (high, med, low) conditions but not in the air condition.

Fig. 2) Regression analysis. Dependent variable: eVAAS "Fear/Discomfort" score.



Relative weight (Beta scores) of separate DSM panic symptoms in predicting the intensity of "Fear/Discomfort" after the inhalation of 0% (air), 9% (low), 17.5% (med), and 35% (high) CO_2.

Factor analysis

The factor analysis extracted and rotated three components – respiratory, cognitive, and neurovegetative – which accounted for over 65% of the variance (Table 3). The respiratory component was mainly characterized by "sensation of shortness of breath" and "feeling of choking". It also included "dizziness", "sweating", and "palpitations". The cognitive component was defined by "fear of dying", "fear of losing control", and "derealization". The neurovegetative component was primarily characterized by "trembling" but also included "chest pain", "chills", "nausea", and "paresthesias".

Regression analysis (2)

The three principal factor scores were then computed as variables. Regression analysis of factor scores as predicting variables (respiratory, cognitive, neurovegetative) showed that the respiratory component was a better predictor of eVAAS than the cognitive and the neurovegetative components (Beta standardized coefficients β=0.541; 0.391; 0.178, respectively). The model explained 45% of the total variance (R=0.67).

Table 3) Factor analysis of Panic Symptom List scores.

	RESPIRATORY	COGNITIVE	NEURO VEGETATIVE
Shortness of breath	0.856		
Feeling of choking	0.845		
Dizziness	0.606		0.475
Sweating	0.582		
Palpitations	0.551		0.419
Fear of dying		0.906	
Fear of losing control		0.868	
Derealization		0.744	
Trembling			0.781
Chest Pain	0.421		0.756
Chills			0.697
Nausea			0.649
Paresthesias			0.642

Principal component analysis of DSM panic symptoms (performed only in 35% CO_2 condition). Three clusters are extracted: respiratory, cognitive, and neurovegetative. Only factor scores >0.4 are shown**.**

Discussion

In the present study, we analyzed the phenomenology of the CO_2-induced reaction in healthy volunteers. The symptoms reported by the subjects after the inhalations could be clustered into three distinct components. This confirms the first hypothesis that panic symptoms can be

subgrouped in discrete clusters. The clusters were labelled “respiratory”, “neurovegetative”, and “cognitive”, according to the most prominent symptoms. The respiratory cluster was determined mainly by the feelings of shortness of breath and choking (second hypothesis). Moreover, a strong relation was demonstrated between the respiratory symptoms and the subjective feeling evoked by the CO_2 challenge, which, according to the DSM criteria for PA, was labelled “fear or discomfort”. Our third hypothesis was that respiratory symptoms were the best predictor of the subjective disturbance defined in the DSM IV criteria for panic. The CO_2-induced emotion was specifically correlated with the respiratory sensations, and these symptoms were indeed the best predictors of the subjective fear/discomfort.

Fear and discomfort are two separate emotional entities but they appear together in the definition of Panic Attack provided by DSM-IV (APA, 2000). Consistently, in order to adhere as strictly as possible to the current psychiatric nosology, the subjects were asked to indicate the amount of the subjective experienced feeling, either it was fear or discomfort. The subjects were not instructed to specifically recognize the type of emotion they were feeling, in order to minimize a cognitive interpretation of their emotional experience.

The factor analysis of the present study extracted three components. The respiratory component was mainly characterized by shortness of breath and choking; it also included dizziness, sweating, and palpitations. The neurovegetative component was primarily characterized by trembling, but also included chest pain, chills, nausea, and paresthesias. The cognitive factor was characterized by fear of dying, fear of losing control, and derealization.

To the best of our knowledge, this is the first factor analysis performed on CO_2-induced panic symptoms in healthy subjects. Previous reports on PD patients (Argyle *et al*, 1989; Briggs *et al*, 1993; Cox *et al*, 1994; de Beurs *et al*, 1994; Meuret *et al*, 2006; Shioiri *et al*, 1996) generally agreed on several clusters, one representing a respiratory component. The similarity between our clusters, extracted in a normal sample, and clusters found in studies on PD patients supports the idea that the CO_2 challenge in healthy volunteers may be a valid model to study the phenomenology of clinical panic.

Specifically, the most recent analysis by Meuret *et al.* (2006) found a cardio-respiratory cluster that is strikingly similar to our respiratory cluster with regard to the presence of

dyspnea, feeling of choking, and palpitations. However, it is slightly different for other symptoms, like dizziness and sweating, which, in Meuret's PD patient sample, were included in the autonomic/somatic cluster. Instead, in our study sweating, dizziness, and palpitations loaded on the respiratory component. Sweating showed high coefficients of correlations to many other panic symptoms (Table 2) and might represent an unspecific reaction, which occur in presence of general distress, including cases of respiratory distress. Palpitations may be considered related to a cardio-respiratory dimension, since changes in heart rate and cardiac output can be secondary to changes in respiratory drive. Dizziness may be considered part of a hyperventilation cluster therefore it may be explainable why it loaded on the RESPIRATORY component in our study. Moreover, dizziness might also be secondary to a transient cerebral hypoperfusion, secondary to bradycardia. Unfortunately we do not have any physiological measurement in the present study however, according to the studies of Argyropoulos *et al.* (2002) and Kaye *et al.* (2005) on the effects of CO_2 in healthy volunteers, the expected cardiovascular reaction to CO_2 consists in a bradycardic response accompanied by an activation of the sympathetic system with an increase in blood pressure. The bradycardia appears to be regulated independently of the pressor response and most likely involves the direct activation of brainstem vagal nuclei by CO_2. Palpitations might also derive from the same changes in heart rate and the consequent activation the baroreceptor reflex. Therefore, since both dizziness and palpitations are strongly related to the activation of the autonomic system, it may be explainable why a substantial secondary loading for dizziness and palpitations was also present on the neurovegetative component (Table 3).

The majority of factor analyses performed on clinical samples found that fear of dying loaded with the cardio-respiratory symptoms (Argyle *et al*, 1989; Briggs *et al*, 1993; Cox *et al*, 1994; de Beurs *et al*, 1994; Meuret *et al*, 2006), while in the present analysis it was the core symptom of the cognitive cluster. Such a difference may reflect a different cognitive elaboration of cardio-respiratory symptoms by PD patients compared to a non-clinical sample in that most PD patients tend to "catastrophize" bodily symptoms whereas healthy subjects are not expected to have developed this cognitive pattern.

As far as the third hypothesis is concerned, the most important finding of the study was that of the respiratory component as the best predictor of the negative affective response to CO_2

challenges. CO_2 inhalation significantly induced almost every panic symptoms listed in the DSM IV. However, we showed that subjective fear/discomfort was specifically associated with an increase in respiratory symptoms. Feeling of choking, shortness of breath, dizziness, and palpitations had the highest scores (mean score>1 in the high condition) and the highest correlation coefficients with regard to the CO_2-evoked subjective disturbance, represented by the eVAAS score. Yet, while dizziness and palpitations were related to the eVAAS score in every condition, including the air condition, the respiratory symptoms had high correlation coefficients specifically in the CO_2-enriched conditions, that is only in the low, medium, and high conditions.

Most of the clinical studies referred to above have provided support for the idea that respiratory symptoms are clinically relevant in PD. The respiratory subtype of panic has a specific profile of pharmacological sensitivity, it is associated with familial risk of PD, and it may predict a greater severity of illness (Biber *et al*, 1999; Briggs *et al*, 1993; Nardi *et al*, 2003; Nardi *et al*, 2006a; Nardi *et al*, 2006b; Onur *et al*, 2007). Epidemiological studies indicate that the presence of respiratory disorders, such as COPD (Goodwin *et al*, 2002), increases the likelihood of developing PD. Moreover, the link between panic and respiration is strengthened by the finding that PD patients have higher levels of irregularity and complexity of respiratory patterns (Caldirola *et al*, 2004).

Some authors have suggested that not only respiratory symptoms, but also the cognitive cluster, represented by catastrophic misinterpretations, seems to correlate quite well with the severity of PD (Cox *et al*, 1991; McNally *et al*, 1995; Telch *et al*, 1989; Vickers and McNally, 2005). However, as the authors themselves admit, a possible limitation of those studies is the retrospective self-report design, which may have overestimated cognitive symptoms. In addition, even if the severity of cognitive misinterpretations does correlate with the severity of the disorder, the direction of causality from cognitive misinterpretations to the severity of PA has not yet been convincingly demonstrated. It is perfectly conceivable that the development of catastrophic misinterpretation may be secondary to the severity of attacks, rather than the severity of attacks being secondary to the self-generated misinterpretations.

Having demonstrated, that in experimental panic, subjective fear/discomfort is more correlated to respiratory sensations than to cognitions, we provide support for the idea that respiratory control, rather than cognitions, plays a major role in the pathophysiology of panic. An interesting hypothesis put forward by Klein some years ago (Klein, 1993; Preter *et al*, 2008) inferred that PAs might derive from an abnormal "suffocation alarm", an evolved, survival-oriented, inner physiological mechanism that erroneously signals the lack of useful air and the danger of impending suffocation, thus leading to urgency to breathe.

If, indeed, from an evolutionary point of view, panic emerged as an emotion to alert the subject of a biological danger, then it seems logical that a subjective sensation of panic (or discomfort) goes along with the severity of respiratory sensations. This is exactly what we observed in our results, which perfectly fit the core idea proposed by Klein, but also fit the function of emotions as expressed by such authors as Damasio (1999) and Panksepp (1998).

One apparent peculiarity of our results may need further comment. We observed that, in the high condition, "feeling of choking" was the best predictor of the fear/discomfort score while "sensation of shortness of breath" appeared to be a poor predictor (Fig. 1). The opposite behavior was observed in the low condition. The importance of choking in predicting the eVAAS score increased with the dose while the importance of shortness of breath decreased. Recently, Ietsugu and coworkers (2007) examined the relationship between the severity of PA and individual symptoms reported by a clinical sample. Shortness of breath was a predictor of moderate severity of PAs, while choking was a good predictor of high severity. Our results in experimental panic seem to be in line with Ietsugu's results in clinical panic. It may be proposed that the feeling of choking is more closely related to a stronger sense of suffocation, which is more likely to be evoked by the higher doses, while the sensation of shortness of breath may reflect milder symptoms of dyspnea.

In conclusion, the present study explored the phenomenology of an experimental model of panic in healthy volunteers. We found that the discrete clusters of symptoms observed in this study were similar to those elicited in naturally occurring panic. Consistent with the idea that respiration plays a crucial role in the pathophysiology of panic, we found that respiratory symptoms best predicted the subjective state defined in the DSM IV criteria for panic.

Chapter IV

Multidimensional characterization of respiratory symptoms in CO_2-elicited panic in healthy volunteers

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Abstract

We characterized the emotional response to CO_2 in healthy volunteers using a multidimensional instrument that measures the sensorial and affective components of respiratory discomfort, the Multidimensional Dyspnea Profile (MDP). All the dimensions of respiratory discomfort were affected by the CO_2 challenge. We found that the rating of respiratory discomfort was strongly correlated to the intensity of the CO_2–induced panic feeling, showing a higher predictive power relative to that of all non-respiratory DSM-IV panic symptoms. MDP scores were able to accurately discriminate between responders and non-responders to the CO_2 challenge. By demonstrating that a scale rating respiratory discomfort could be successfully applied as a tool to evaluate the subjective response to CO_2, we provide support to the idea that respiratory symptoms are a central phenomenon in experimental panic induced by CO_2

Introduction

Panic Disorder (PD) and respiration are closely inter-related. Respiratory discomfort (or dyspnea), is a central phenomenon in the experience of a Panic Attack (PA) (Schruers *et al*, 2004b). A "respiratory" subtype of panic, characterized by the prevalence of respiratory symptoms, is associated to specific clinical features, a specific profile of pharmacological sensitivity, and a higher sensitivity to carbon dioxide (CO_2) inhalation (Biber *et al*, 1999; Briggs *et al*, 1993; Freire *et al*, 2010; Horwath *et al*, 1997). As a further confirmation of the link between panic and respiration, epidemiological studies evidenced high comorbidity of PD and respiratory disorders (asthma, chronic obstructive pulmonary disease) (Goodwin *et al*, 2002). It has been also hypothesized that the neurobiological mechanisms underlying PD may involve respiratory control (Battaglia *et al*, 2005; Esquivel *et al*, 2010; Klein, 1993; Preter *et al*, 2008).

The hypersensitivity to CO_2 displayed by PD patients and their first-degree relatives is well recognized (Coryell *et al*, 2006; Perna *et al*, 1996; Roberson-Nay *et al*, 2010; van Beek *et al*, 2005). In addition to its use as a disease-specific biomarker of PD, the CO_2 challenge can be used as an experimental model of PAs in healthy volunteers, as increasing concentrations of CO_2 dose-dependently induce a negative affect, compliant with the definition of PA, also in healthy individuals (Griez *et al*, 2007). This finding suggests that high doses of CO_2 can elicit panic in healthy subjects, as moderate doses do in PD patients.

The panic response induced by CO_2 challenge has been classically assessed following the definition of PA in the DSM diagnostic criteria (APA, 2000). Subjects are asked to rate their subjective feeling (fear or discomfort) and their panic symptoms, which include both somatic and cognitive symptoms. It has been previously showed in healthy volunteers that PA symptoms, elicited by high-dose CO_2 inhalation, can be grouped in distinct dimensions, namely respiratory, neurovegetative, and cognitive, and that the respiratory dimension was the best predictor of the emotional response to CO_2 (Colasanti *et al*, 2008). These findings suggest that a careful analysis of the CO_2-induced respiratory symptoms is important to elucidate the phenomenology of the emotion elicited by the CO_2 challenge.

Respiratory discomfort consists of qualitatively distinct sensations, and it has been well demonstrated that it comprises sensorial and affective dimensions that can be measured as separate entities (Lansing *et al*, 2009). There is experimental evidence that different stimuli produce distinct kinds of respiratory discomfort (Banzett *et al*, 2008). Therefore, a careful assessment of the CO_2-induced respiratory symptoms should be multidimensional, and should take into account both the affective and sensorial components.

The scope of the present study is to characterize a focused and specific, multidimensional instrument to measure respiratory discomfort, in terms of its potential application as a tool to rate the subjective response to CO_2 challenges. The Multidimensional Dyspnea Profile (MDP) has been designed to measure different dimensions of respiratory discomfort: unpleasantness, sensory intensity, and emotional response (Banzett *et al*, 2008).

We aim to 1) assess the response to acute high dose CO_2 inhalation using the MDP in healthy volunteers and 2) test whether and how accurately the rating of respiratory discomfort can predict the intensity of the panic-like feeling induced by CO_2, and discriminate between responders and non-responders to the challenge. Furthermore, we want to characterize how the different dimensions of respiratory discomfort differentially contribute in discriminating the response to acute CO_2 inhalation. We hypothesize that the MDP, a respiratory rating instrument, will be able to accurately predict the emotional response to CO_2. As the response to CO_2 is recognized as a valid human model of PAs, this would support the hypothesis that respiratory symptoms are central in the phenomenology of panic.

Materials and methods

Subjects

The study is part of a larger research aimed to investigate the neurobiological mechanisms underlying the response to CO_2 inhalation. The other results, concerning the effects of dietary manipulations on CO_2-induced emotion, have been published elsewhere (Colasanti *et al*, 2011). Healthy volunteers were recruited among students or staff, through advertisements from within Vijverdal Psychiatric Hospital in Maastricht and from the Maastricht University. The ethics committee of Maastricht University approved the study and the subjects have been paid for their participation to the experiment. A complete description of the study was given both orally and in written form to the subjects. The volunteers underwent collection of medical history and physical examination. Inclusion criteria were: an age ranging between 18 and 65 years and a good present and past physical and mental condition, based on the results of a structured psychiatric interview (Mini International Neuropsychiatric Interview) (Sheehan *et al*, 1998) performed by a physician. Exclusion criteria were: current psychopharmacological or psychological treatment, presence of alcohol, substance or caffeine–related disorders, excessive smoking (>10 cigarettes a day), respiratory or cardiovascular disease, hypertension (diastolic>100 mmHg; systolic >170 mmHg), personal or familiar history of cerebral aneurysm, pregnancy, and epilepsy. Individuals were also excluded if they reported common specific fears or if they had a history of PAs, or a history of PD in a first-degree relative. Those who met the inclusion and exclusion criteria and who provided written informed consent were included in the study.

Carbon Dioxide challenge

The procedure was standardized for all subjects and for all sessions and was performed according to the protocol for 35% CO_2 inhalation used at the Maastricht Academic Anxiety Center (Griez *et al*, 1987a). A gas mixture containing 35% CO_2 / 65% O_2 was delivered through a nasal-oral exercise self-administration facemask using a double vital capacity inhalation technique. Before the challenge was started, the inspired vital capacity of every

subject was evaluated using an analogue respirometer (Wright respirometer Mark 20) connected to the self-administration mask. The same respirometer measured the gas volume delivered at each inhalation. Baseline inspired vital capacity with a double breath of air was measured on each occasion, and a test breath was considered adequate if it was more than 80% of the baseline vital capacity. The subjects were then given the self-administration mask and told to hold it in their hands and to exhale as deeply as they could. A double-breath inhalation of the 35% CO_2 / 65% O_2 mixture was then delivered to the subjects (Griez *et al*, 2007). They were asked to take a maximal inspiration through the self-administration facemask and to make a complete expiration outside the facemask, immediately followed by a second maximal inspiration through the facemask following the same procedure. At the end of the second inhalation, the subjects were asked to hold their breath for 4 seconds to enhance the alveolar gas exchange.

Assessments

We used a visual analogue scale for affect (VAAS) labelled 'fear or discomfort', ranging from 0 (no fear/discomfort at all) to 100 (the worst imaginable fear/discomfort). The participants were instructed to indicate the amount of the subjective disturbance, in case of feeling either fear or discomfort following an established procedure (Colasanti *et al.*, 2008).

Panic symptoms were evaluated using the Panic Symptom List (PSL-IV) (Schruers *et al*, 2000b). This consists of a questionnaire listing 13 items, each representing one of the DSM-IV symptoms (palpitations; sweating; trembling; sensations of shortness of breath or smothering; feeling of choking; chest discomfort; nausea or abdominal distress; feeling dizzy, lightheaded, or faint; derealisation or depersonalization; fear of losing control; fear of dying; paresthesias; chills or hot flushes). The participants were asked to rate the intensity of each symptom from 0 (absent) to 4 (very intense). The total scores thus ranged from 0 to 52.

To assess dyspnea, we used the MDP (Banzett *et al*, 2008). This instrument is designed to measure immediate unpleasantness (A1), sensory qualities (SQ), and emotional responses (A2). A1 is an individual item, while SQ and A2 are composed of categories. The scales

comprising all integers from 0 to 10, equally spaced, were presented for ratings. For the A1 item, in addition to the numbers, words descriptive of magnitude and dimension were ranged along the scale to improve consistency among subjects; the score 0 was associated with the description "neutral", scores between 1 and 3 were associated with "slightly unpleasant", 4 and 5 were described "annoying", while between 6 and 8 were "distressing". The upper end of the SI scale (scores 9 and 10) was labelled ''maximum,'' whereas the upper end of the A1 scale was labelled ''unbearable".

The five categories of SQ were: "My breathing requires muscle work or effort"; "I am not getting enough air, I feel hunger for air, or, I am smothering"; "My breathing requires mental effort or concentration"; "My chest and lungs feel tight or constricted"; "I am breathing a lot; (breathing rapidly, deeply, heavily)". Subjects rated how much of each sensation quality they felt, from 0 ("none") to 10 ("as intense as I can imagine").

A2 was rated through a list of five negative emotions: depression, anxiety, frustration, anger and fear and two positive emotions, happiness and contentedness. Subjects rated how much of each emotion they experienced on a scale ranging from 0 ("none") to 10 ("the most I can imagine").

All the assessments were administered 10 minutes before (pre-CO_2) and immediately after CO_2 challenge (post-CO_2). Post-CO_2 scores indicated the worst moment experience by subjects after inhaling the gas mixture.

Data analysis

The statistical analyses were performed using SPSS 17.0. All the data are presented as means ± SD. Changes in VAAS, PSL, and MDP scores were calculated with the formula: Δ scores= post-CO_2 – pre-CO_2 scores.

A MDP total score was generated by adding A1 and each SQ scores to the A2 scores related to negative emotions (depression, anxiety, frustration, anger and fear). The two positive emotions, happiness and contentedness, were not included in the MDP Total Score as they

are unlikely to contribute to the negative affect associated to respiratory discomfort. The positive emotions have been included by the authors of the scale to help subjects to pay attention to the items (personal communication).

A "responder" to CO_2 inhalation was defined accordingly to previously applied conservative criteria (Griez et al., 2007), which indicate as responder a subject reporting both Δ VAAS scores >50 and Δ PSL subscores >1 in at least four PSL symptoms.

The accuracy of each rating scales (MDP and PSL subscales) in discriminating responders from non-responders has been determined using Receiving Operator Characteristics (ROC) curves. The ROC curves have been obtained by plotting the true positive rates (sensitivity of the test) and false positive rates (1 – specificity), with each point of the curve corresponding to different true positive and false positive rates. The overall accuracy of the test performance has been summarized by the area under the curve (AUC) obtained by connecting the points. The AUC value could range from 0.5, indicating no better than chance test performance (sensitivity and specificity = 50%), to 1.0, with sensitivity and specificity = 100%, indicating perfect test discrimination. The significance of the differences between the ROC curve and the true diagonal (Reference Line) has been tested assuming a non-parametric distribution. The null hypothesis was that AUC of the variable of interest was equal to that of the Reference Line (AUC=0.5).

The statistical analysis was aimed to test the following hypothesis:

1. CO_2 inhalation results in significant increases in MDP Total Score and subscales scores.
2. MDP Total Score significantly predicts Δ VAAS fear/discomfort scores and significantly discriminate responders from non-responders to the challenge.
3. MDP Total Score predictive power (in predicting Δ VAAS and identifying responders) is at least equal to that of PSL.

Additionally, we aimed to test which sensorial and affective dimensions of dyspnea significantly discriminate responders from non-responders in an optimal way.

Accordingly, we have performed the following statistical analyses:

1. A one-tailed T-test for Paired Samples to compare pre- and post-CO_2 MDP Total and MDP subscales scores.
2. Linear regression analysis using Δ VAAS scores as dependent variable and either
    a. each of Δ MDP A1, SQ, and A2 scores *or*
    b. each of individual Δ PSL scores (with or without respiratory symptoms)

    as predicting variables.
3. ROC curves to classify responders and non-responders generated for Total Δ MDP Scores and Total Δ PSL Scores, respectively. The significance of the difference between their AUC and Reference Line has been tested.
4. ROC curves to classify responders and non-responders generated for each Δ MDP A1, SQ and A2 scores. The significance of the difference between their AUC and Reference Line has been tested.

Results

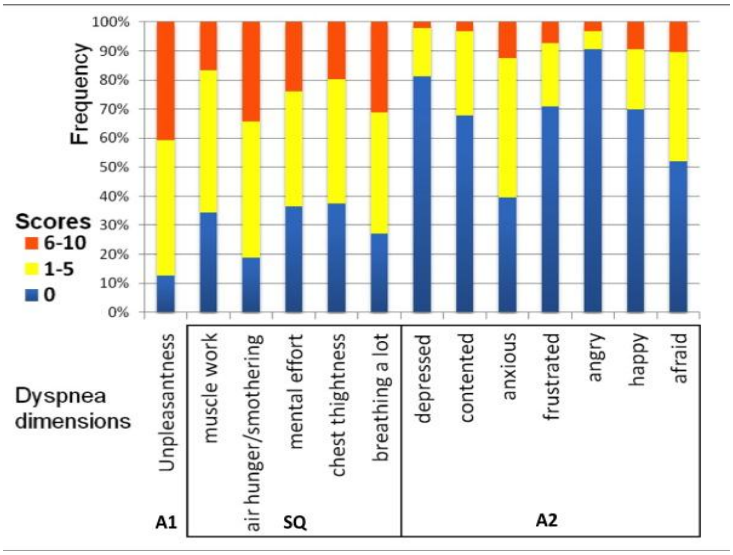
We analyzed data from 96 CO_2 challenges performed in 57 subjects (30 males) included in the study. The mean age was 24.6 ± 6.1 years.

CO_2 inhalation induced a significant change in all MDP ratings, demonstrated by a significant post-CO_2 vs pre-CO_2 increase in Total scores, A1, all SI subscales ($p<0.0001$), and all A2 subscales scores with negative valence [anxiety, fear, frustration ($p<0.0001$), anger ($p<0.001$), and depression ($p<0.05$)], and by a significant decrease in the A2 subscale scores with positive valence [happiness ($p<0.0001$) and contentedness ($p<0.05$)]. The frequency distribution of post-CO_2 MDP subscale ratings is represented in Fig. 1. With regard to "unpleasantness" (A1) scores, the experience was rated at least "distressing" (post-CO_2 A1 scores≥6) in 39 cases (40.6%).

There was a significant increase in VAAS scores (mean Δ VAAS=38.7 ± 25.3), total PSL scores (mean total Δ PSL score=11.5 ± 6) and all individual PSL scores ($p<0.0001$) except

"nausea or abdominal distress" and "fear of dying". In 33 cases (34.4%) the subjects were responders to the CO_2 challenges. Among those who were responders, 72.7% rated post-CO_2 MDP A1≥6 ("distressing or unbearable").

Fig. 1) Frequency of post-CO_2 MDP scores.



MDP scores 6 to 10 were labelled with the descriptors "distressing" (6 to 8), or "unbearable" (9 and 10). MDP scores 1-5 were labelled with the descriptors "slightly unpleasant" (1 to 3), or "annoying" (4 and 5). The score 0 was labelled with the descriptor "neutral". A1: unpleasantness; SQ: sensory qualities; A2: emotional responses.

Linear regression

The linear regression analysis showed that the regression model which included all Δ MDP sub-scores as predictor variables, was significantly able to predict Δ VAAS scores (R^2=0.553; F=7.793; p<0.0001). The model which included all PSL scores as predictor variables was less effective in predicting Δ VAAS scores (R^2=0.391; F=4.051; p>0.0001), and the only significant regression coefficient displayed by the PSL regressors was that of

shortness of breath. The same model, after removing PSL respiratory symptoms (sensation of shortness of breath and feeling of choking), was a relatively weak predictor of Δ VAAS scores (R^2=0.255; F=2.610; non-significant), and there were no statistically significant regression coefficients among PSL symptoms.

ROC curves

ROC curves for Total Δ MDP and Total Δ PSL scores are presented in Fig. 2. Both AUCs were significantly different from that of the asymptotic curve (p<0001), indicating that the null hypothesis is rejected. AUCs are similar between the two classifiers (0.815 and 0.792, respectively for Total Δ MDP and Total Δ PSL scores), indicating that they are equally effective in discriminating between responders and non-responders to CO_2.

With regard to the SQ scores (Table 2, Fig. 4), all the dimensions were significantly different from the asymptotic curve in discriminating between responders and non-responders, however the SQ with the highest discriminating power was air hunger/sensation of smothering or suffocating.

ROC curves for individual affective dimensions (Δ MDP A1 score and individual Δ MDP A2 scores) are showed in Table 1 and Fig. 3. Δ MDP A1 (unpleasantness) displays the highest AUC, followed by anxiety and fear, and frustration (0.835, 0.759, 0.722, 0.674, respectively). Depression, happiness, anger and contentedness' AUCs were not significantly different from that of the asymptotic curve.

Fig. 2) ROC curve of Total Δ MDP and Total Δ PSL scores.

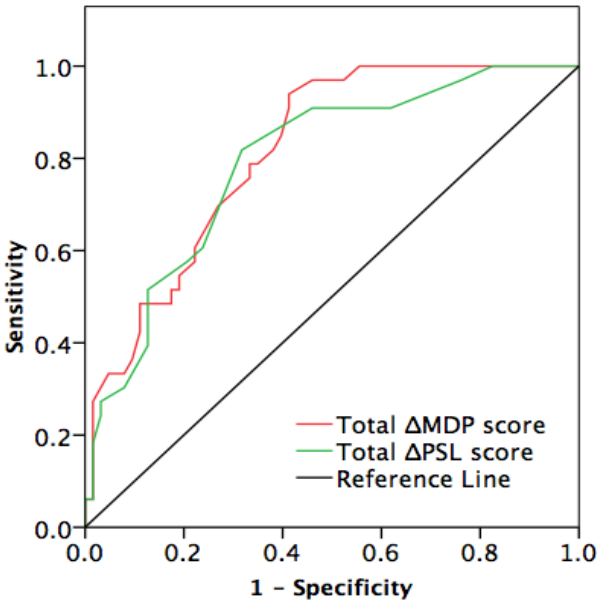


Table 1. AUC of individual affective dimensions of respiratory discomfort (Δ MDP A1 score and individual Δ MDP A2 scores).

Affective dimensions	AUC	SE	Sig.	95% CI	
unpleasantness	.835	.044	.000	.748	.921
depression	.550	.063	.425	.427	.673
anxiety	.759	.053	.000	.656	.863
contentedness	.597	.061	.120	.477	.716
frustration	.674	.061	.005	.555	.793
anger	.596	.063	.124	.472	.720
happiness	.511	.063	.865	.388	.633
fear	.722	.057	.000	.611	.833

Fig. 3) ROC curve of individual affective dimensions of respiratory discomfort (Δ MDP A1 score and individual Δ MDP A2 scores).

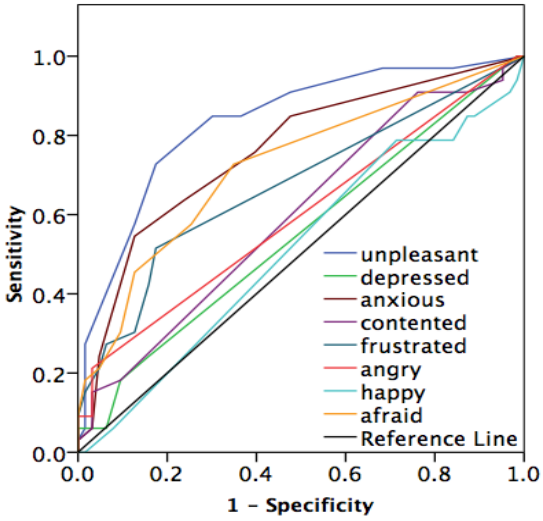
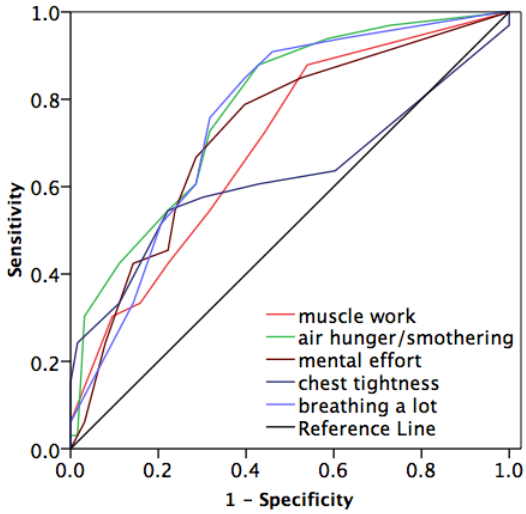


Table 2. AUC of individual sensory qualities of respiratory discomfort (Δ MDP SQ scores)

Sensory Qualities	AUC	SE	Sig.	95% CI	
Muscle Work	.697	.055	.002	.590	.805
Air Hunger/Smothering	.779	.048	.000	.685	.874
Mental effort	.726	.054	.000	.619	.833
Chest tightness	.626	.067	.043	.494	.758
Breathing a lot	.757	.050	.000	.660	.855

Fig. 4) ROC curve of individual sensory qualities of respiratory discomfort (Δ MDP SQ scores).



Discussion

We characterized the respiratory discomfort in response to CO_2 inhalation, an acute panicogenic challenge, in healthy volunteers using the multidimensional scale MDP. By investigating whether a scale rating respiratory discomfort could be successfully applied as a tool to evaluate the subjective response to CO_2, we aimed to support the idea that respiratory symptoms are a central phenomenon in experimental panic.

We have found that 1) all the MDP dimensions were strongly affected by the CO_2 challenge; 2) the rating of respiratory discomfort was associated with the intensity of the CO_2 –induced panic feeling, showing a higher predictive power relative to that of all non-respiratory DSM-IV panic symptoms; 3) the rating of respiratory discomfort was able to discriminate between responders and non-responders to the CO_2 challenge; 4) the respiratory dimensions that better

discriminated responders from non-responders were unpleasantness among the affective dimensions, and sensation of air hunger/smothering among the sensorial dimensions.

If all the published literature on the relationship between panic and CO_2 challenges is right (Griez *et al*, 1998; Griez *et al*, 2007; Papp *et al*, 1997) in claiming that the CO_2 challenge is a valid experimental model of panic, then it appears from the present findings that a respiratory instrument designed by physiologists is better in grasping panic than a psychiatric instrument rating all the DSM-IV symptoms of panic (the PSL). This leads to the thought that panic is essentially a respiratory phenomenon, at least from a phenomenological point of view.

These findings also suggest that MDP can be implemented to monitor the subjective response to CO_2 challenges. The accurate assessment of respiratory discomfort induced by CO_2 provides important information to investigate the phenomenology of CO_2-induced emotion and to further understand the relationship between respiratory sensations and panic.

The results of this study replicate and extend our previous findings obtained by clustering CO_2–induced panic symptoms, which indicated that a cluster of respiratory symptoms of panic could be extracted in the experimental panic similarly to the naturally occurring panic. It was also shown that the respiratory dimension was the best predictor of the affective response to CO_2 inhalation (Colasanti *et al*, 2008).

In the present study, we observed that the CO_2 inhalation significantly affected the rating of all respiratory dimensions measured with MDP and that the immediate feeling induced by CO_2 (unpleasantness of respiration, or A1) was rated as at least “distressing” in more than 40% of the cases and in more than 70% of the responders to the challenge. MDP was strongly associated to the intensity of the affective response to CO_2 (measured with VAAS) and discriminated between responders and non-responders to the challenge at least with the same accuracy as the PSL did. Furthermore, a regression model including all MDP sub-scales was more powerful in predicting the affective response than a model including the other, non-respiratory, panic symptoms. This is consistent with another study that examined dimensions of dyspnea evoked by 35% CO_2 challenge in PD patients and demonstrated that

breathing effort and sense of suffocation discriminated patients who did and did not have CO_2-induced PAs (Perna *et al,* 2004b).

These results are in line with the clinical evidence pointing that respiratory symptoms are central in the phenomenology and clinical course of PD. The presence of prominent respiratory symptoms is associated with a distinct profile of pharmacological sensitivity (Briggs *et al*, 1993), a familial risk of PD (Horwath *et al*, 1997; Nardi *et al*, 2003; Nardi *et al*, 2006a), a longer duration and a greater severity of illness (Biber *et al*, 1999; Onur *et al*, 2007). Other clinical features displayed by PD patients with prominent respiratory symptoms include a higher sensitivity to CO_2 inhalation (Abrams *et al*, 2006; Biber *et al*, 1999; Nardi *et al*, 2006a; Valenca *et al*, 2002), more past traumatic suffocation experiences and respiratory diseases (Bouwer *et al*, 1997; Verburg *et al*, 1995b), higher levels of anxiety sensitivity, and more symptoms of the panic-agoraphobic spectrum (Onur *et al*, 2007). Epidemiological studies indicate that the presence of respiratory disorders, such as chronic obstructive pulmonary disease (Goodwin *et al*, 2002a), increases the likelihood of developing PD.

We found that the affective dimension of respiratory discomfort that best discriminated between responders and non-responders to the CO_2 challenge was the immediate experience of unpleasantness, rather than emotional responses which require a more extensive cognitive evaluation, such as anxiety, fear and frustration. The latter are "secondary" emotions, which involve the broader context of a person's life experience and personal situation, and other cognitive inputs (Lansing *et al*, 2009). Instead, unpleasantness is an immediate "feeling state", more primitive, non-reflective, and more directly related to the homeostatic urge of breathing. We hypothesize that this finding is consistent with the nature of the CO_2-induced emotion.

Increased CO_2 levels are sensed by central and peripheral CO_2/H^+ chemoreceptors, which provide rapid feedback to the brainstem respiratory control system concerning the adequacy of alveolar ventilation relative to metabolism and also monitor the balance of arterial CO_2, cerebral blood flow, and cerebral metabolism. Recent findings demonstrated that central chemoreception is a distributed property of different groups of midbrain neurons, which also include midbrain serotonergic neurons (Severson *et al*, 2003). These have been proposed to

mediate non-respiratory responses to hypercapnic acidosis, such as intense feeling of anxiety. Also, the amygdala, a key region in processing affective stimuli, is intrinsically CO_2/H^+ chemosensitive, and its chemosensitive properties regulates the expression of fearful behavioural responses in rodents (Ziemann *et al*, 2009). Based on these evidences, it appears that the *emotional* brain respond directly to hypercapnic acidotic stimuli. Behavioural evidence in humans, showing that acutely increased CO_2 levels dose-dependently elicit an emotional distress (Griez *et al*, 2007), and neuroimaging evidence, showing limbic neural activation correlating with CO_2-induced distress (Liotti *et al*, 2001), support this idea. The emotion elicited by CO_2 could represent the subjective-affective element of an instinct critical for survival, that is the urge of breathing. Denton proposed that hunger for air is an instance of a primal or primordial emotion, similar in nature to those emotions experienced during states of pain, thirst, and hunger, when the sensation component is interoceptor-driven and linked to the basic homeostatic functions of life (Denton *et al*, 2009). It is not surprising that in our study the emotional response to CO_2 was better described as an immediate and non-reflective "feeling state" of unpleasantness rather than a more complex secondary type of emotion.

We found that all the sensorial dimensions discriminated between responders and non-responders to the challenge, however the dimension which better predicted the response was "not getting enough air, hunger for air, smothering". Klein proposed the existence of an inborn CO_2-driven alarm in humans, aimed to protect the organism in case of impending suffocation (Klein, 1993; Preter *et al*, 2008). It is conceivable that panic is part of the human emotional repertoire and is elicited by suffocation cues to signal to the body the need of more air. Our findings support this theory.

In the present study, the CO_2 inhalation induced a significant change in all affective dimensions of MDP, including depression and anger, which are not directly related to panic. Other experimental studies have indicated that emotions other than panic can be elicited by CO_2 inhalation. In a study in PD patients, depressive and aggressive symptoms were provoked by CO_2 inhalation, particularly in those subjects also suffering from Major Depression (Overbeek *et al*, 2005). Prolonged 7.5% CO_2 breathing has been described to elicit reductions in happiness and relaxation ratings, and increases in fear, anxiety, tension,

nervousness, irritability, and worrying in healthy volunteers (Bailey *et al*, 2005). Consistently with the profile of provoked symptoms 7.5% CO_2 has been used as an experimental model of GAD, rather than PD (Bailey *et al*, 2007).

Conclusions

We have characterized the emotional response to CO_2 using a multidimensional instrument which measures the sensorial and affective components of respiratory discomfort. Our results suggest that MDP could be successfully used to evaluate the subjective response to CO_2 and that could be extended to assessments of clinical populations. Also, additional research is worth to test whether it is a sensitive instrument to detect the effects of panicolytic agents on the subjective response to CO_2.

In conclusion, the ability of a respiratory instrument to capture the expression of experimental panic is paradigmatic of the important link existing between respiration and panic.

Chapter V

Effects of tryptophan depletion and tryptophan loading on the affective response to high-dose CO_2 challenge in healthy volunteers

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Abstract

It has been reported that in Panic Disorder (PD), tryptophan depletion enhances the vulnerability to experimentally induced panic, while the administration of serotonin precursors blunts the response to challenges. Using a high dose Carbon Dioxide (CO_2) challenge, we aimed to investigate the effects of acute tryptophan depletion (ATD) and acute tryptophan loading (ATL) on CO_2-induced panic response in healthy volunteers.

Eighteen healthy volunteers participated in a randomized, double-blind placebo-controlled study. Each subject received ATD, ATL, and a balanced condition (BAL) in separate days, and a double-breath 35% CO_2 inhalation 4.5 h after treatment. Tryptophan (Trp) manipulations were obtained adding 0 g (ATD), 1.21 g (BAL), and 5.15 g (ATL) of l-tryptophan to a protein mixture lacking Trp. Assessments consisted of a Visual Analogue Scale for Affect (VAAS) and Panic Symptom List (PSL). A separate analysis on a sample of 55 subjects with a separate-group design has also been performed to study the relationship between plasma amino acid levels and subjective response to CO_2.

CO_2-induced subjective distress and breathlessness were significantly lower after ATD compared to BAL and ATL ($p<0.05$). In the separate-group analysis, Δ VAAS scores were positively correlated to the ratio Trp:ΣLNAA after treatment ($r=0.39$; $p<0.05$).

The present results are in line with preclinical data indicating a role for the serotonergic system in promoting the aversive respiratory sensations to hypercapnic stimuli (Richerson, 2004). The differences observed in our study, compared to previous findings in PD patients, might depend on an altered serotonergic modulatory function in patients compared to healthy subjects.

Introduction

In patients affected by Panic Disorder (PD), acute tryptophan depletion (ATD) enhances the response to a number of panicogenic agents. This effect of ATD has been shown in studies which used inhalation of 35% and 5% Carbon Dioxide (CO_2) (Miller *et al*, 2000; Schruers *et al*, 2000a) and infusion of flumazenil (Bell *et al*, 2002; Davies *et al*, 2006) as challenges to induce panic, but no effect has been observed using infusion of cholecystokinin tetrapeptide (CCK-4) (Toru *et al*, 2006). Pre-treatment with 5-hydroxytryptophan (5-HTP), the immediate precursor of serotonin (5-HT), has been shown to blunt the response to 35% CO_2 challenge, indicating a "protective" effect on experimentally induced panic (Schruers *et al*, 2002b). These findings seem to indicate that, in PD patients, fear or anxiety provoked by some panicogenic challenges is negatively correlated to the availability of 5-HT precursors.

Studies in healthy volunteers yielded inconclusive findings: reports showed that ATD failed to modify the panicogenic effects of CCK-4 (Koszycki *et al*, 1996) as well as the effects of 5% CO_2 inhalation (Miller *et al*, 2000) or the Read rebreathing test (Struzik *et al*, 2002). Two studies have tested the effects of 35% CO_2 in tryptophan (Trp) depleted subjects, one showing an increase of CO_2-induced neurovegetative symptoms but not subjective anxiety (Klaassen *et al*, 1998), while the other did not find any significant difference between ATD condition and placebo with regard to CO_2-provoked subjective effects (Hood *et al*, 2006). Administration of 5-HTP in healthy volunteers was shown to reduce CCK-4-induced panic attacks and panic-related cognitive symptoms specifically in females (Maron *et al*, 2004) but had no effects on the response to CO_2 (Schruers *et al*, 2002b).

The discrepancies observed between PD patients and healthy controls, in terms of the effects of ATD on the subjective response to challenges might be due to the relative low sensitivity exhibited by healthy subjects to panicogenic agents. We have previously showed in healthy volunteers that the inhalation of CO_2 dose-dependently induces panic-like symptoms, and that high doses of CO_2 (double-breath of 35% CO_2) in healthy subjects might be as effective as moderate doses of CO_2 are in PD patients (Griez *et al*, 2007; Schruers *et al*, 2004b). Here we intended to perform a study in healthy subjects using a high dose CO_2 challenge in order to test whether we can reproduce in a non-clinical population the same modulating effects of

5-HT manipulation observed in PD patients on experimental panic response; for this purpose, we investigated the effects of ATD on CO_2-induced panic response in healthy volunteers.

Additionally, since the Trp suppletion studies with panicogenic challenges conducted in healthy subjects gave inconclusive results (Maron *et al*, 2004; Schruers *et al*, 2002b), we also tested the effects of acute tryptophan loading (ATL) on subjective response to CO_2 challenge.

Material and Methods

Subjects

Healthy volunteers were recruited amongst students or staff, through advertisements from within the Vijverdal Psychiatric Hospital Maastricht *(*Mondriaan Zorggroep) and throughout the Maastricht University locations. The Medical Ethics Committee of the Academic Hospital Maastricht and Maastricht University approved the study and the subjects were paid for their participation in the experiment. After a complete description of the study, a written informed consent was obtained from the subjects. The volunteers underwent collection of medical history and physical examination. Inclusion criteria were age between 18 and 65 years and a good present and past physical and mental condition. The latter was established using a structured psychiatric interview (Mini International Neuropsychiatric Interview) performed by a physician. Exclusion criteria were current psychopharmacological or psychological treatment, recent alcohol intake, substance or caffeine-related disorders, excessive smoking (>10 cigarettes a day), respiratory or cardiovascular disease, hypertension (diastolic>100 mmHg; systolic>170 mmHg), personal or familiar history of cerebral aneurysm, pregnancy, and epilepsy. Individuals were also excluded if they reported common specific fears or if they had a history of panic attacks, or a history of PD in a first-degree relative.

Design

Subjects were randomized in double-blind placebo-controlled cross-over study design. The subjects received three different gelatin-based mixtures (GBM) on three different days, in a randomized order, to induce respectively the ATD condition, a balanced condition (BAL) and the ATL condition. On each day, after GBM administration, they underwent a double-breath CO_2 challenge.

A separate analysis has been conducted on a larger sample of subjects in a randomized separate-group design, in order to investigate the relationship between the ratio Trp to the sum of large neutral amino acids in plasma (Trp:ΣLNAA) and the subjective response to CO_2. Subjects were randomly assigned to one of the three groups: ATD condition, BAL condition, and ATL condition. This sample also included subjects who were enrolled in the cross-over study for which only the first study day was taken into consideration in the analysis.

Procedure

The subjects arrived at the clinic after an overnight fast. Blood was drawn to measure plasma Trp and LNAA levels. GBM were administered in the morning at about 9.30. After drinking the GBM, the subjects remained on the ward and were allowed to read or watch a nature documentary on video. Subjects had ad libitum access to mineral water but they were asked to refrain from eating and drinking any xanthine beverages. At 4.5 h after GBM administration other blood samples were collected to monitor plasma Trp and LNAA levels. Ten minutes later, the subjects underwent a double-breath inhalation of a gas mixture containing 35% CO_2 and 65% O_2.

Gelatin-based mixtures

The gelatin consists of a hydrolysate collagen-protein comprising the entire range of amino acids in the form of peptides, but completely lacking Trp. After administration, these peptides are decomposed into amino acids, and the mechanism of depletion is identical to

that of the "classic" amino acid mixture (Sambeth *et al*, 2009). The GBM was kindly provided by PB Gelatins (Tessenderlo Group, Belgium) in form of powder. Amino acid composition of the GBM can be found in Table 1. The drink was prepared mixing 100 g of the powder with 200 ml water at 50-70°C. The drink was kept refrigerated at 4°C and then kept at room temperature for the 30 min before administration. The three GBM were identical in composition except that 1.15 g of l-tryptophan and 5.15 g of l-tryptophan were added to the mixtures for the BAL and ATL conditions, respectively. No l-tryptophan was added for the ATD condition. The three GBM had the same colour and taste.

Table 1. Amino-acid spectrum

Amino acid spectrum (typical weight % on ds protein)	
Alanine	8.4
Arginine	7.7
Aspartic Acid/Asparagine	4.5
Cysteine	0.0
Glutamic Acid/Glutamine	10.0
Glycine	23.3
Histidine	0.9
Hydroxylysine	1.5
Hydroxyproline	12.3
Isoleucine	1.2
Leucine	2.6
Lysine	3.3
Methionine	0.9
Phenylalanine	1.6
Proline	13.7
Serine	3.4
Threonine	1.9
Tryptophan	0.0
Tyrosine	0.6
Valine	2.2

Carbon Dioxide challenge

The 35% CO_2-inhalation procedure was performed in accordance to a standardized protocol developed at the Maastricht Academic Anxiety Center (Griez *et al*, 1987a; Griez *et al*, 1998). A gas mixture containing 35% CO_2/65% O_2 was delivered through a nasal-oral exercise self-administration facemask using a double vital capacity inhalation technique. Before the challenge, the inspired vital capacity of every subject was measured using an analogue respirometer (Wright respirometer Mark 20) connected to the self-administration mask. The same respirometer measured the gas volume delivered at each inhalation. The inspired vital capacity with a double breath of air was measured on each occasion, and a challenge was considered adequate if it was more than 80% of the baseline vital capacity. The subjects were then given the self-administration mask and asked to exhale as deeply as possible. They were asked to take a maximal inspiration through the mask and to make a complete expiration outside the mask, immediately followed by a second maximal inspiration. At the end of the second inhalation, the subjects were asked to hold their breath for 4 seconds, to enhance the alveolar gas exchange, and finally make a complete expiration outside the mask again.

Amino acid analysis

Samples for determination of plasma amino acid levels were taken at baseline (T0) and 4.5 h after GBM administration (T1). Blood (10 ml) was collected by venepuncture in sodium heparin tubes at each time point, immediately after the rating of subjective assessments. After collection, the blood samples were immediately centrifuged at 4°C (10 min at 4000 rpm). Subsequently, 100 µl of plasma was mixed with 8 mg of sulphasalicyl acid and frozen at −80°C until the amino acid analysis was performed (van Eijk *et al*, 1993). Plasma amino acids were determined using a fully automated high-performance liquid chromatography (HPLC) system after precolumn derivatization with *o*-phthaldialdehyde (OPA). OPA-AA derivates of the amino acids were quantified with fluorescence detection. The concentrations of plasma amino acids were expressed as µmol/l (van Eijk *et al*, 1993). The ratio of total Trp:ΣLNAA (LNAA: large neutral amino acids, i.e., tyrosine, phenylalanine, leucine,

isoleucine and valine) at baseline and 4.5 h after GBM were used as endpoints to monitor changes in Trp availability.

Assessments

Rating scales to assess panicogenic effects of CO_2 challenge were chosen with reference to the definition of Panic Attack in DSM-IV TR diagnostic criteria (APA, 2000). We used a visual analogue scale for affect (VAAS) labelled "fear or discomfort", ranging from 0 (no fear/discomfort at all) to 100 (the worst imaginable fear/discomfort). The participants were instructed to indicate the amount of the subjective disturbance, in case of feeling either fear or discomfort following an established procedure (Colasanti *et al*, 2008).

Panic symptoms were evaluated using the Panic Symptom List (PSL-IV) (Schruers *et al*, 2000a). This consists of a questionnaire listing 13 items, each representing one of the DSM-IV TR symptoms (i.e., palpitations; sweating; trembling; sensations of shortness of breath or smothering; feeling of choking; chest discomfort; nausea or abdominal distress; feeling dizzy, lightheaded, or faint; derealization or depersonalization; fear of losing control; fear of dying; paresthesias; chills or hot flushes). The participants were asked to rate the intensity of each symptom from 0 (absent) to 4 (very intense). The total scores thus ranged from 0 to 52.

VAAS and PSL-IV were administered at baseline (T0; pre-GBM administration), 1.5 H hrs (T1), 3 hrs (T2), 4.5 h post-GBM administration (T3), and after CO_2 challenge (Post-CO_2). Post-CO_2 scores indicated the worst moment experienced by the subjects after inhaling the gas mixture.

Mood states were measured with the shortened 32-item validated version of the Dutch translation of the Profile of Mood States Scale (POMS) (Wald and Mellenbergh, 1990), which consists of 5 mood scales (depression, tension/anxiety, vigor, anger/hostility, and fatigue). The POMS was administered at T0, T1, T2, and T3. Subjects were asked to rate the scale according to how they felt at that moment.

Data analysis

All the data are presented as mean ± standard deviation (SD). Percentage changes in Trp:ΣLNAA ratio (Δ% Trp:ΣLNAA ratio) after GBM (T3) compared to baseline (T0) were calculated by the formula T3-T0/T0×100. CO_2-induced changes in VAAS and PSL scores were expressed as Δ scores (obtained by the formula: POST-CO_2 scores - T3 scores). In the cross-over sample, all data were analyzed with analysis of variance (ANOVA) for repeated measures with time and treatment condition as within-subjects factors. The effects of time × treatment condition interaction were studied to investigate the influence of GBM condition (ATD, BAL, ATL) on the subjective response to CO_2 measured with VAAS and PSL scores. The analysis of VAAS scores has been repeated after controlling for gender, and the interaction time × treatment condition × gender has been studied using ANOVA per repeated measures.

To investigate the influence of GBM conditions on POMS scores, the effects of time per se and time × treatment condition interaction were studied with ANOVA per repeated measures.

If indicated by a significant ANOVA condition effect, time effect, or time × condition interaction effect, a subsequent evaluation on difference between individual conditions and between individual time points was done by within-subject repeated contrasts. The level of significance was set at 0.05. For the separate-groups analysis the principal statistical analysis consisted of Spearman's non-parametric correlation between ΔVAAS scores and Trp:ΣLNAA levels. A partial correlation analysis between these two variables was repeated after controlling for gender. Moreover, baseline differences between treatments/groups were analyzed by one-way ANOVA for continuous variables (age, weight, Trp:ΣLNAA ratio, VAS and PSL scores) and chi-square for non-parametric variables (gender distribution).

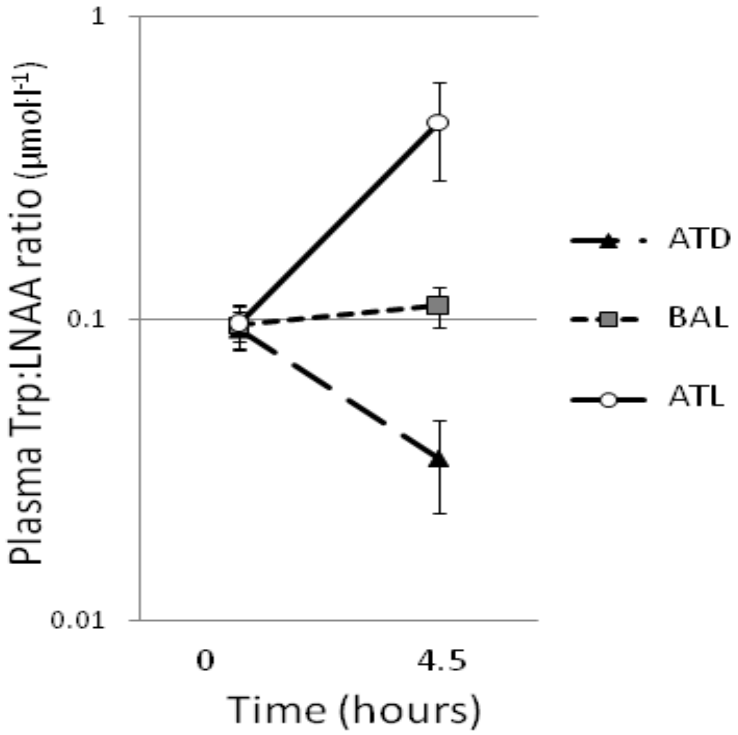
Results

Eighteen healthy volunteers (10 male) completed the cross-over study (mean age 25±5.5 years). One subject was excluded because she reported nausea and vomiting after the administration of GBM.

Amino acid levels

Plasma amino acid levels are presented in Fig.1. Total Trp:ΣLNAA ratio at T0 did not significantly differ between treatment conditions (F=0.88, p=0.42). A significant time X condition interaction was found, with Δ% Trp:ΣLNAA ratio (from T0 to T3) being different between conditions (F=106.6; p<0.0001). ATD resulted in a decrease of 61.35±15.6% in Trp:ΣLNAA ratio compared to baseline, while ATL resulted in an increase of 361.53±154.05%. A 17.76±19% post-GBM increase was found in the BAL condition. Within-subject contrasts evidenced significant differences between changes in Trp:ΣLNAA ratio between ATD and BAL, between ATL and BAL, and between ATD and ATL (F=106.6; p<0.0001). In three cases of ATD condition, we observed a decrease in Trp:ΣLNAA ratio <50% and in one case of BAL condition, an increase in Trp:ΣLNAA ratio >50% was observed. For all the other subjects, we found Trp:ΣLNAA changes >50% in ATD condition, <50% in balanced condition, >100% in the ATL condition. The analyses reported in the following sections have also been performed after exclusion of those three subjects, obtaining the same results as with the complete sample.

Fig. 1) Plasma amino-acid levels



Plasma Trp:ΣLNAA ratio at baseline and 4.5 h after treatment across conditions: acute tryptohan depletion (ATD), balanced condition (BAL), acute tryptophan loading (ATL); time × condition interaction: $p<0.0001$; Trp:ΣLNAA ratio (µmol/l) is presented on logarithmic scale.

Subjective measures

VAAS scores (fear/discomfort) between conditions, at different time points, are presented in Fig. 2. There were no significant differences in VAAS scores at T0, T1, T2, and T3 between conditions. No effect of GBM administration per se was observed on VAAS scores, as no significant difference was found between any of pre-CO_2 challenge time points (T3, T2, T1) and T0, in any condition. CO_2 inhalation was followed by an increase in VAAS scores in all the conditions (F=57.49; p<0.0001). A significant time X condition interaction was found,

indicating that ΔVAAS scores were significantly lower in ATD compared to BAL and ATL (33.89±26.18 vs 43.78±25.5 and 44.17±23.88, respectively; F=5.79 and F=6.58, p<0.05). No significant differences were found between BAL and ATL conditions. The findings remained identical after controlling for gender and no effects of time X gender X condition interaction have been found.

Fig. 2) VAAS scores for fear/discomfort

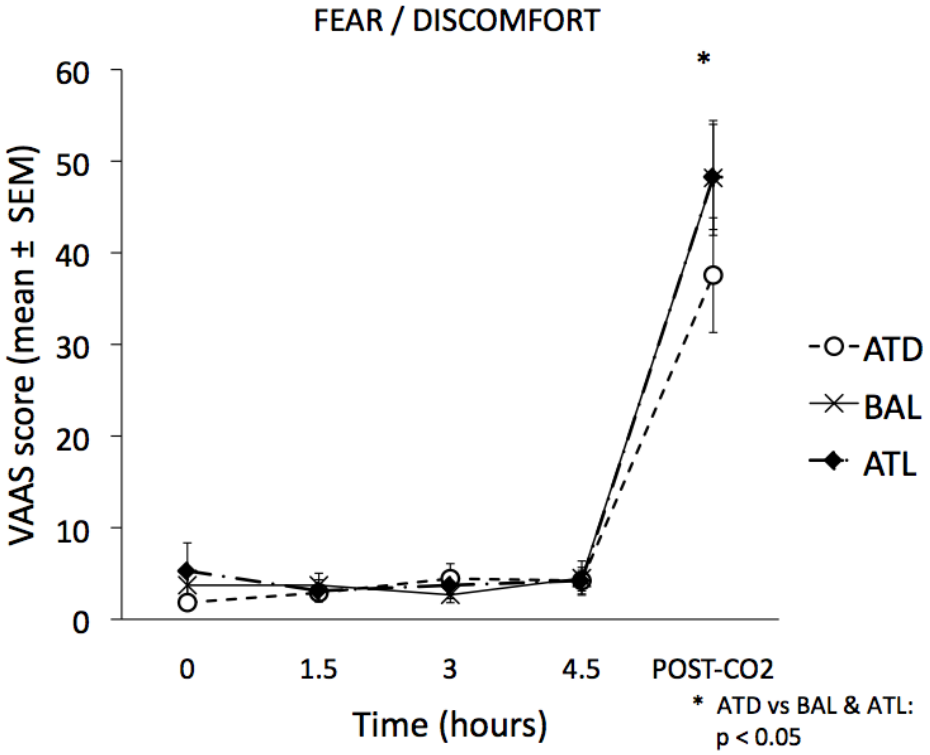


Fig. 2) VAAS scores for fear/discomfort at 0, 1.5, 3, 4.5 h after treatment, and after CO_2 inhalation, across treatment conditions: acute tryptohan depletion (ATD), balanced condition (BAL), acute tryptophan loading (ATL); change in VAAS scores was significantly lower in ATD compared to BAL and ATL (time × condition interaction: p<0.05).

There were no significant differences in PSL scores between conditions at any time point. Total PSL scores did not change at T1, T2, and T3 compared to T0, but significantly increased after CO_2 inhalation, relative to T3 (F=97.51; p<0.0001). ΔPSL scores were similar between conditions (ATD: 11.11±6.35; BAL: 11.06±4.87; TL: 11.22±4.32; NS). Analyzing individual PSL items separately, the only significant effect of treatment conditions on ΔPSL scores was evident for the item "sensation of shortness of breath", indicating that ΔPSL scores after CO_2 were lower in ATD condition than in BAL and ATL conditions (F=4.11; p<0.05) (Fig. 3).

Fig. 3) PSL scores for sensation of shortness of breath

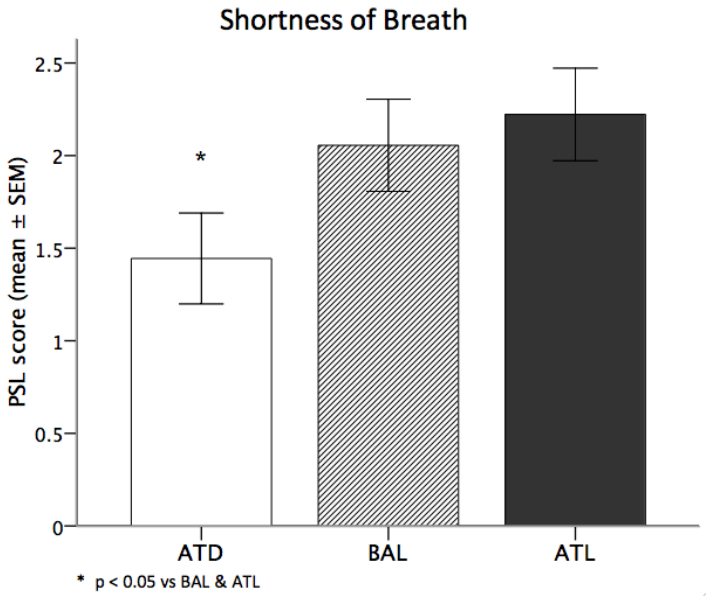


Fig. 3) Change in PSL scores for shortness of breath after CO_2 inhalation relative to T3, across treatment conditions: acute tryptohan depletion (ATD), balanced condition (BAL), acute tryptophan loading (ATL); change in PSL scores was significantly lower in ATD compared to BAL and ATL (time × condition interaction: p<0.05).

No order effect was found and ΔVAAS and ΔPSL scores were not affected by the order of administration of GBM conditions.

There was a significant effect of time on POMS-vigor (p<0.0001) and POMS-tension (p<0.05) scores, and within-subjects contrasts indicated that POMS-vigor scores were significantly higher at T0 relative to T1 (p<0.01), and significantly lower at T2 compared to T3 (p<0.01). POMS-tension scores were higher at T0 compared to T1 (p<0.05). There was no significant effect of the time × treatment condition interaction on POMS scores.

Separate–group study

Fifty-five volunteers were enrolled in the separate group study. Treatment groups consisted in 19 subjects (8 male; age: 24.84±5.33 years) in ATD condition, 19 subjects (10 male; age: 23.32±4.46 years) in BAL condition, and 17 subjects (10 male; 24.65±5.32) in ATL condition. Age, gender distribution, and weight did not significantly differ between groups. Trp:ΣLNAA ratio at T0 was similar between conditions and baseline VAAS scores and PSL scores did not significantly differ between groups either. The relationship between Trp:ΣLNAA ratio and ΔVAAS scores is presented in Figs. 4 and 5. ΔVAAS scores were positively correlated to Δ% Trp:ΣLNAA ratio and Trp:ΣLNAA ratio at T3 [Spearman's rho= 0.395 (p<0.005) and 0.381 (p<0.005), respectively], indicating that higher VAAS scores were associated with larger increases in the ratio Trp:ΣLNAA. No correlation was found between total PSL scores and Δ% Trp:ΣLNAA ratio or Trp:ΣLNAA ratio at T3.

Fig. 4) Correlation between Δ% Trp:ΣLNAA ratio and ΔVAAS scores

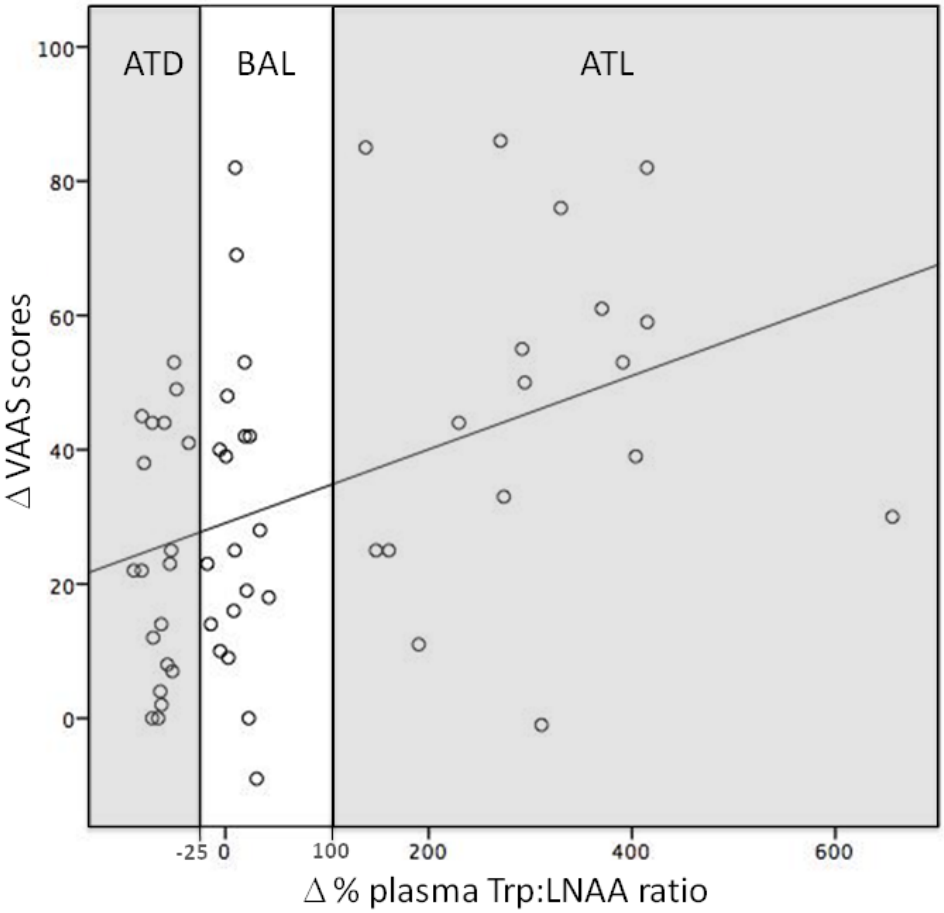


Fig. 4) Relationship between % change in Trp:ΣLNAA ratio after treatment and change in VAAS scores after CO_2 inhalation; change in VAAS scores after CO_2 inhalation was positively correlated to % change in Trp:ΣLNAA ratio [Spearman's rho= 0.395, p<0.005]

Fig 5) Correlation between Trp:ΣLNAA ratio at T3 and Δ VAAS scores.

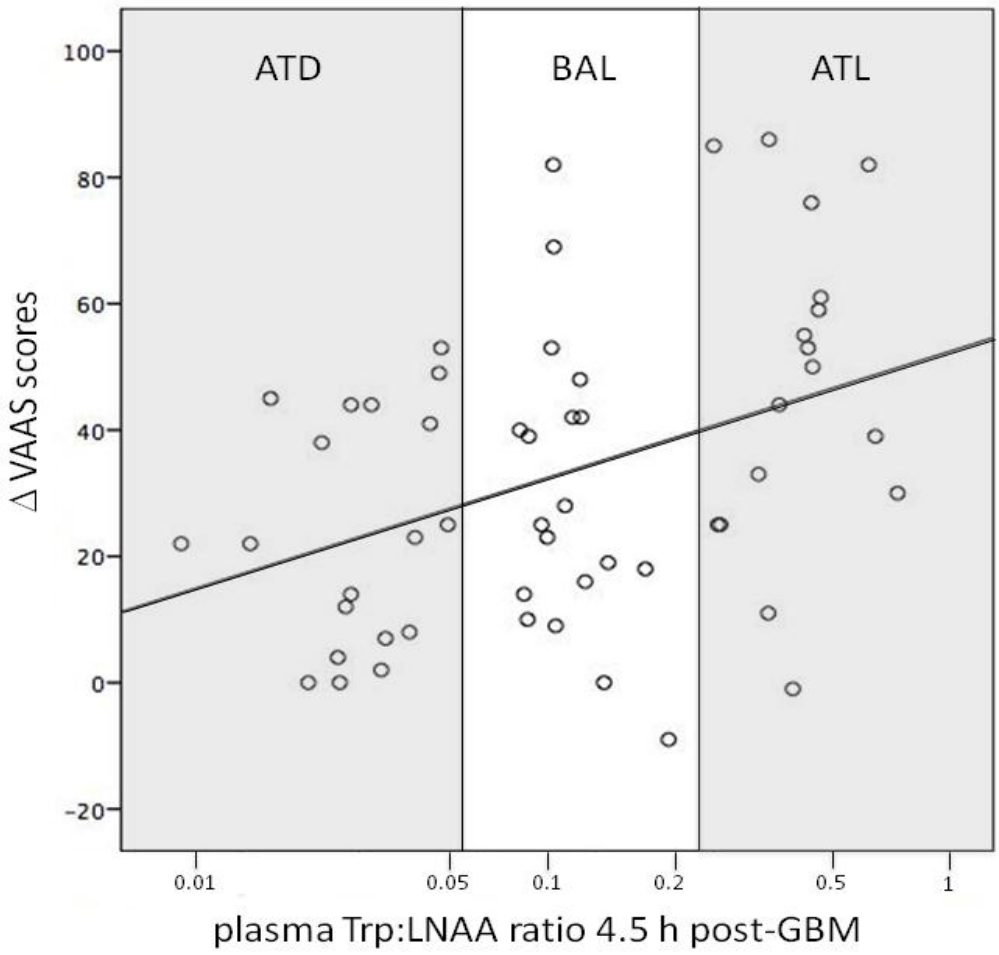


Fig. 5) Relationship between Trp:ΣLNAA ratio at T3 (4.5 h post-treatment) and change in VAAS scores after CO_2 inhalation; change in VAAS scores after CO_2 inhalation was positively correlated to Trp:ΣLNAA ratio at T3 [Spearman's rho=0.381, p<0.005]; Trp:ΣLNAA ratio (μmol/l) is presented on logarithmic scale.

Discussion

To investigate whether the amount of available 5-HT precursors influence the vulnerability to a panicogenic challenge, we tested the subjective response to a 35% CO_2 inhalation after acute Trp depletion, Trp loading, and placebo in healthy volunteers. We found a mild, but significant effect of treatment condition on the subjective scores of fear/discomfort, indicating that ATD is associated to a reduced response to a CO_2 inhalation relative to placebo and ATL. The same effect was also evident for scores on the panic symptom "shortness of breath". The analysis of the other separate individual symptoms and the total composite score of panic symptoms induced by CO_2 did not show any significant difference between conditions. In a separate analysis, we also investigated the relationship between Trp availability (Trp:ΣLNAA ratio) and subjective response to CO_2, as measured by VAAS scores, in a separate-group design. We found a relatively weak, but significant positive correlation between the Trp:ΣLNAA ratio and CO_2-induced changes in VAAS scores, indicating that lower availability of 5-HT precursors is associated to a blunted affective response to CO_2.

These findings are in contrast with previous results found in studies by our laboratory (Klaassen *et al*, 1998; Schruers and Griez, 2004a; Schruers *et al*, 2000a; Schruers *et al*, 2002a; Schruers *et al*, 2002b) and in other CO_2 challenge studies in healthy volunteers (Hood *et al*, 2006) and in PD patients (Miller *et al*, 2000). First, we will discuss potential explanations for the divergent findings observed in this study compared to other studies with inhaled CO_2 and other panicogenic agents in both healthy volunteers and PD patients. Furthermore, we will try to interpret our findings in the context of the complex relationship between panic, 5-HT and CO_2, also in light of the recent data, which suggest that 5-HT neurons are sensitive to acute changes in CO_2 concentration. Methodological issues and limitations relative to the technique used to manipulate 5-HT precursors will be finally addressed.

Tryptophan depletion/suppletion studies in healthy volunteers and PD patients

A large body of evidence suggests that manipulation of 5-HT precursors availability has an influence in modulating panic and anxiety (see review by (Maron *et al*, 2008). However, some of the findings from experimental studies, both in healthy volunteers and PD patients, are divisive and indicate that the role of 5-HT might not be unique.

Hood *et al.* (2006) and Klaassen *et al.* (1998) tested the effects of ATD on the response to single-breath 35% CO_2 challenge in healthy subjects. In a study on 14 healthy volunteers, no significant differences were found in subjective measures between ATD and balanced conditions, although ATD resulted in an increased CO_2-induced elevation of cortisol compared to the balanced condition (Hood *et al*, 2006). Klaassen's study (Klaassen *et al*, 1998), including 15 volunteers, found a significant increase in CO_2-induced neurovegetative symptoms after ATD. In that study sample, mean net increases in total PSL scores and VAAS scores after CO_2 were <7 and <7, respectively (Klaassen *et al*, 1998). Subjective CO_2-induced anxiety was, therefore, very mild, hence indicating that single-breath 35% CO_2 in healthy subjects did not evoke "real" panic. In the present study, using double breath CO_2, we found a more than 30% higher increase in PSL scores and more than 500% higher increase in VAAS scores after CO_2 compared to Klaassen's (1998) study. Average VAAS and PSL scores in the present double-breath 35% CO_2 study are comparable to those normally found in PD patients after single-breath 35% CO_2 (Verburg *et al*, 1998b). Other challenges in healthy volunteers found no significant effect of ATD on responses to anxiogenic challenges. This has been showed using a 5% CO_2 challenge (Miller *et al*, 2000) and the hypercapnic Read rebreathing technique (Struzik *et al*, 2002), a Simulated Public Speaking challenge (Monteiro-dos-Santos *et al*, 2000), and CCK-4 (Koszycki *et al*, 1996). In contrast, a study by (Goddard *et al*, 1995) showed increased nervousness in response to yohimbine challenge after ATD compared to yohimbine alone.

To enhance 5-HT availability in healthy subjects, Maron and coworkers (2004) administered 5-HTP (direct precursor of 5-HT) and used CCK-4 as panicogenic challenge in 32 healthy volunteers. They observed a significant reduction compared to placebo in CCK-4-induced panic attacks and cognitive symptoms only in females whereas in males only a decrease in

somatic symptoms was observed. In Schruers et al.'s study, 5-HTP did not alter the response to 35% CO_2 compared to placebo (Schruers *et al*, 2002b). Taken together, these data and our results indicate that the effects of Trp depletion in healthy volunteers largely depend on the type of challenge and its relative potency, and might be additionally confounded by gender effects.

Studies in PD patients demonstrated that ATD increased the response to single breath 35% CO_2 (Schruers *et al*, 2000a) and 5% CO_2 anxiety (Miller *et al*, 2000), and administration of 5-HTP blunted the response to single breath 35% CO_2 (Schruers *et al*, 2002b)

ATD also increased the response to flumazenil infusion in PD patients successfully treated with selective serotonin reuptake inhibitors (SSRI) (Bell *et al*, 2002; Davies *et al*, 2006) or CBT (Bell *et al*, 2011). In contrast, subjective response to CCK-4 is not influenced by ATD (Toru *et al*, 2006) in SSRI-treated PD patients. Also, ATD did not have significant effects on the subjective response to anxiogenic challenges in OCD (Barr *et al*, 1994; Kulz *et al*, 2007) and in GAD SSRI–treated patients (Hood *et al*, 2010). It is interesting to note that in the latest study of Hood and colleagues (2010), using 7.5% CO_2 challenge, some subjective measures of anxiety ("something bad is going to happen", "anxious", "secure"), seemed to indicate an anxiolytic effect of ATD rather than anxiogenic, however pairways comparison was not significantly different between conditions.

As a further confirmation of the role of 5-HT in modulating experimental panic, serotonergic drugs that are effective in treating Panic Disorder, like SSRI and tryciclics, also reduce the fear that patients with PD experience when they inhale CO_2 (Bertani *et al*, 2001; Bertani *et al*, 1997; Perna *et al*, 2004a; Perna *et al*, 2002; Perna *et al*, 1997; Pols *et al*, 1996b).

In summary, a number of studies in PD indicate that availability of 5-HT precursors is inversely related to vulnerability to CO_2 challenges, which is at odds with the present results. However, overall findings in anxiety disorder patients suggest that the effects of Trp manipulation specifically depend on the diagnosis and the type of anxiogenic challenge.

Panic, 5-HT and CO_2: A complex relationship

Accumulating evidence from clinical and experimental research, and genetic studies suggest a substantial role for the 5-HT system on the neurobiology of PD (Maron and Shlik, 2006). The relationship between 5-HT and panic is complex, as exemplified by the notion that SSRI are effective in reducing panic, but they may exacerbate anxiety during the initial phase of treatment (Sinclair *et al*, 2009). It has been hypothesized that panic is associated to either 5-HT excess (Iversen, 1984) and 5-HT deficit (Deakin and Graeff, 1991). Deakin and Graeff proposed that the 5-HT system plays a dual role in the modulation of anxiety by inhibiting panic responses, but contributing to anticipatory or generalized anxiety. Our findings presented here are not in line with this theory, as in our experimental design increased 5-HT availability did not suppress CO_2-induced panic responses in healthy volunteers. However, recent studies suggest that other factors should be taken into account in understanding the relationship between 5-HT, CO_2 and anxiety: a subset of 5-HT neurons, located in the chemosensitive zone (ventrolateral medulla and raphe) and associated with large arteries, are intrinsically chemosensitive in vitro (Severson *et al*, 2003) and are stimulated by hypercapnia in vivo in unanaesthetized animals (Veasey *et al*, 1995, 1997). Furthermore, experiments using in vivo microdialysis showed that increasing inhaled CO_2 causes an increase in 5-HT release (Kanamaru *et al*, 2007). Interestingly, mice selectively lacking 5-HT neurons display a blunted respiratory response to CO_2, indicating that 5-HT neurons are required for normal central chemoreception (Hodges and Richerson, 2008). Richerson (, (2004) proposed that medullary 5-HT neurons control ventilatory responses to CO_2 and project to areas like forebrain and limbic system that are involved in affective regulation. Taking into account the panicogenic properties of CO_2, we have previously speculated that these neurons could be part of an adaptive protective mechanism alerting the organism against the risk of impending asphyxia (Griez *et al*, 2007). Taken all these preclinical findings together, it appears that acute hypercapnia stimulates serotonergic neurons and 5-HT release and that conversely, disruption of the 5-HT system blunts the neuronal response to CO_2.

Our data are in line with the above preclinical evidence; we have effectively reduced availability of 5-HT precursors and we have used CO_2 as a panicogenic challenge. In

agreement with Hodges and Richerson's (2008) data, we found that depletion of 5-HT precursors blunted the subjective response to CO_2, particularly the respiratory sensations, and Trp availability was positively correlated with the intensity of the subjective responses.

Methodological limitations

Some methodological considerations regard the validity of Trp manipulations to alter 5-HT availability. Trp depletion and Trp loading are relatively easy procedures to rapidly and reversibly change, decrease and increase respectively, the levels of 5-HT precursors (Hood *et al*, 2005). The first step in 5-HT biosynthesis is the conversion of Trp to 5-HTP by Trp hydroxylase. In the brain, this enzyme is only 50% saturated and the rate at which 5-HT is synthesized is limited only by substrate (Trp) availability. The LNAA transport system at the blood-brain barrier has a high affinity for all the LNAAs, including Trp. Therefore, the ratio Trp:ΣLNAA in plasma is generally used to predict the availability of Trp to the brain. A large body of literature provides evidence that manipulations of the levels of Trp in plasma results in a substantial and parallel alteration of 5-HT synthesis in the brain and availability of 5-HT and its metabolite in humans and animals (Biggio *et al*, 1974; Carpenter *et al*, 1998; Gessa *et al*, 1974; Leathwood, 1987; Lieben *et al*, 2004; Williams *et al*, 1999). However, an altered release of 5-HT efflux (thought to reflect synaptic release, i.e., 5-HT neuronal activity) was only reported after chronic Trp depletion (Fadda *et al*, 2000) or after ATD in combination with 5-HT reuptake inhibition (Bel and Artigas, 1996), but not after ATD alone (van der Plasse *et al*, 2007). In vitro studies suggest that an increase in Trp availability determines dose-dependent changes in 5-HT release under conditions of increased serotonergic neuronal activity but not on basal output of 5-HT (Sharp *et al*, 1992; Wolf and Kuhn, 1986).

We assume that the alterations in the Trp:ΣLNAA ratio of the present study were followed by changes in brain 5-HT availability and synthesis in the same direction. However, at present, we cannot assure that Trp depletion and Trp loading actually influenced 5-HT release. Nevertheless, the possibility exists that the manipulations in the present study did

affect 5-HT neuronal release, based on the above-mentioned notions that acute hypercapnia stimulates serotonergic neurons and induces 5-HT release, and that Trp manipulations seem to be effective in altering 5-HT release only in stimulated neurons.

A number of other methodological issues need to be addressed. To manipulate brain Trp availability, the studies in healthy volunteers of both Klaassen *et al*. (1998) and Hood *et al.* (2006) included the use of the classic amino-acid mixture (Young *et al*, 1985), the former including an addition of carbohydrates and fat. In these studies, similarly to ours, ATD appeared to be as effective and resulted in a significantly decrease of plasma Trp levels. However, the magnitude of the depletion seems difficult to compare with that in our study due to some methodological differences: in the study of Klaassen *et al.* (1998), no baseline Trp plasma levels were collected, and in the study of Hood *et al.* (2006), free Trp plasma levels instead of total TRP levels were used for calculation of the Trp:ΣLNAA ratio. It is still debated whether total Trp or free Trp levels in plasma are the most reliable indirect measures of brain Trp availability (Pardridge, 1998). Therefore, methodological differences might account for the divergent findings in our study compared to previous studies in healthy volunteers.

Conclusions

It is recognized that 5-HT system plays a complex role in the regulation of the panic responses to CO_2, as different serotonergic mechanisms can coexist, either inhibiting panic (Deakin *et al*, 1991) or promoting the aversive respiratory sensations to hypercapnic stimuli. The differences observed in our study in healthy volunteers, compared to previous findings in PD patients, might depend on the different relative contribution of these mechanisms in different populations.

Chapter VI

The affective response to CO_2: a true primal emotion?

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The present thesis is based on a series of studies investigating the phenomenological characteristics and pharmacological modulation of the emotion induced by Carbon Dioxide (CO_2) administration in healthy humans. Before we provide the conclusive remarks on the studies related to the characterization of the CO_2-induced emotion, presented in chapters 2 to 5, it is worth to step back, and carefully address the most delicate and sensitive question of the thesis: "did CO_2 really evoke an emotion?".

This is a question of crucial importance in relation to our studies. We based our investigation on the hypothesis that CO_2-induced emotion may constitute an instance of primal (or primordial) emotion. However, if it's relatively easy to find evidence supporting the 'primordiality' of response to CO_2 (i.e. its ancient phylogenetic roots, the adaptive survival value, the deep subcortical neural processing, etc.), it is not similarly immediate to find satisfactory arguments to support the statement that the subjective response to CO_2 indeed represents an emotion.

With this problem in mind, we focused our attention on the nature of the emotional response to CO_2 in the first part of the thesis, with Chapter 1 reviewing a large number of cases of acute exposure to CO_2 to search any hint suggesting an 'affective' component in the subjective hypercapnic responses, and with Chapter 2 directly investigating the emotional response to CO_2 in an experimentally-controlled setting.

These studies confirmed our hypothesis as they pointed out that CO_2 does induce a dose-dependent affective response, observable in healthy human subjects, as well as in clinical populations. However, it is necessary to acknowledge a potential important caveat pertinent to those studies, which relates to the definition of emotion.

How do we define emotion? Which criteria do we apply to establish that CO_2 induced a real emotion?

In our studies, to test our hypothesis that the subjective response to CO_2 was a real emotion, we used rating scales based on DSM-IV criteria for panic attack, a clinical state characterized by an intense emotional component. We argued that if we could find evidence that CO_2 induces a subjective experience similar to panic, and assuming that panic is an emotion, than

it was logical to believe that that experience qualified as an emotion as well. Nevertheless, we should be cautious in using this reasoning as the unique unequivocal proof that what we were measuring was an emotion indeed. In our studies we have applied a limited range of assessments (Visual Analogue Scales and Panic Symptom List), and although part of them were indeed reflective of emotions, these instruments may have not been sufficient to undoubtedly state that the subjective state elicited by CO_2 is an emotion. We need first to clearly identify a set of criteria defining an emotion, and only then apply these to the observations of our studies, to test if the subjective response to CO_2 satisfies the criteria that such definition implies.

Moreover, we need to formulate a null hypothesis that needs to be more specific and plausible that just stating the CO_2-induced response is not an emotion. If the experience induced by the inhalation of CO_2 is not an emotion, what could it be instead?

Based on our knowledge of the physiology of response to CO_2, there is no doubt that the inhalation of CO_2 is associated to an experience of increased breathing, secondary to a chemosensor-driven ventilatory reflex. Hence, without an affective component the subjective response to CO_2 would be purely the experience of arousal, increased ventilation and other physiological phenomena driven by chemosensors. In other words, the null hypothesis could be formulated as follows:

> "The subjective response to CO_2 is merely the experience, or the perception, of a physiological reflex driven by interoceptors".

Defining an emotion

Providing a definition of emotion is difficult, and according to some authors a definition of emotion, which could be even superficially acceptable, does not exist at all (Mandler, 1984). Often the term emotion is defined with a reference to a list: anger, disgust, fear, joy, etc. Kleinginna and Kleinginna categorized 92 different definitions from the literature and 9 sceptical statements related to the definition of emotion (Kleinginna and Kleinginna, 1981),

looking for a consensual definition. They propose that "the emotion is a complex set of interactions among subjective and objective factors, mediated by neural/hormonal systems, which can (a) give rise to affective experiences such as feelings of arousal, pleasure/displeasure; (b) generate cognitive processes such as emotionally relevant perceptual effects, appraisals, labelling processes; (c) activate widespread physiological adjustments to the arousing conditions; and (d) lead to behaviour that is often, but not always, expressive, goal-directed, and adaptive". Oatley describes emotions as characteristic mental states that normally happen in response to identifiable conditions of elicitation, have distinctive aspects and recognizable consequences (Oatley, 1992). In 1890, William James, that much influenced current theories in affective neuroscience and philosophy of mind, such as those by Jaak Panksepp (Panksepp, 1998) and Antonio Damasio (Damasio, 1999), hypothesized that emotion is *just* the feeling of bodily changes as they occur in response to a relevant stimulus (James, 1890). These various definitions underline different characteristics of the triggers and the circumstances that accompany emotions. However, they do not tell us much about what is the mental experience taking place during emotion, other than describing it in generic terms such as "feeling of arousal, pleasure/displeasure" (Kleinginna *et al*, 1981), "characteristic mental state" (Oatley, 1992), "feeling of bodily changes" (James, 1890).

In a paper published in 2002, Cabanac focused on the characteristics of the mental experience that takes place during emotions and applied a four-dimensional mathematical description of emotion (Cabanac, 2002), following up a definition of consciousness previously formulated using a similar model (Cabanac, 1996). Cabanac proposes that any mental experience (Ψ) taking place in consciousness could be described by a mathematical equation that includes the dimensions: duration (axis t), quality (axis x), intensity (axis y), and pleasure/displeasure (axis z) (Equation 1).

$$\Psi = f(x[t], y[t], z[t]) \qquad \text{Equation 1}$$

With regard to the axis x (qualitative dimension), what emotions have in common is that they are "aroused by exposure of the subject to situations more or less related to motivation […] resulting in a behaviour oriented towards, or away, from the stimulus" (Cabanac, 2002).

Stimuli can be sensorial or resulting from imagination or memory. With regard to the y-axis (intensive dimension), the author proposes that high intensity is a prerequisite for emotions. This seems in agreement with the view on emotion of other authors, for instance Griffiths ("emotion is an irruptive motivational complex in higher cognition") (Griffiths, 1997) and, even more clearly, with Denton's idea of primal emotions ("[…] they can reach intensity which gives total occupancy of the stream of consciousness and plenipotentiary power over behaviour") (Denton, 2006). Intensity is a necessary but not sufficient condition, as a mental event of high intensity does not qualify as an emotion if it does not have a hedonic component. That component, the hedonic or pleasure/displeasure dimension, represents the axis z. Damasio proposed that the pleasure is the dimension of consciousness that motivates the subject towards useful behaviours (Damasio, 1994). The hedonic dimension serves to rank priorities and ensure that the most urgent motivation has first access to the behavioural common path. In other words, pleasure is a sign of usefulness. If emotion is an integrative response whose biological function is primarily homeostatic, we expect all emotions to be highly hedonic, either positively or negatively, and indeed no mental state commonly recognized as emotion is hedonically indifferent (for instance fear, anger, desire, sadness, disgust, love, joy, etc.). All these emotions are intense mental events aroused by exposure to situations related to motivation, either positive or negative but all resulting in a behaviour oriented to, or away from, the stimulus. Finally, as any other mental event, emotion has a limited duration (axis t), which is closely related to the stimulus that aroused it.

In conclusion, emotion is a mental experience and possesses the four dimensions of consciousness: quality, intensity, hedonicity, and duration. Cabanac summarizes and concludes that emotion is any mental experience with high intensity and high hedonicity (Cabanac, 2002).

Based on the above, the null hypothesis can be refined as:

> "The subjective response to CO_2 is merely the experience, or the perception, of a physiological reflex driven by interoceptors and is characterized by either low intensity or low hedonicity."

Application of the definition of emotion to the subjective response to CO_2

We take for granted that the subjective response to CO_2 is a mental state. We then examine each of the dimensions individually, and test if each criterion of the definition of emotion provided by Cabanac (Cabanac, 2002) is applicable to our studies.

Axis X: **Quality**

Definition*: Emotions are aroused by exposure of the subject to stimuli, sensorial or mental, more or less related to motivation.*

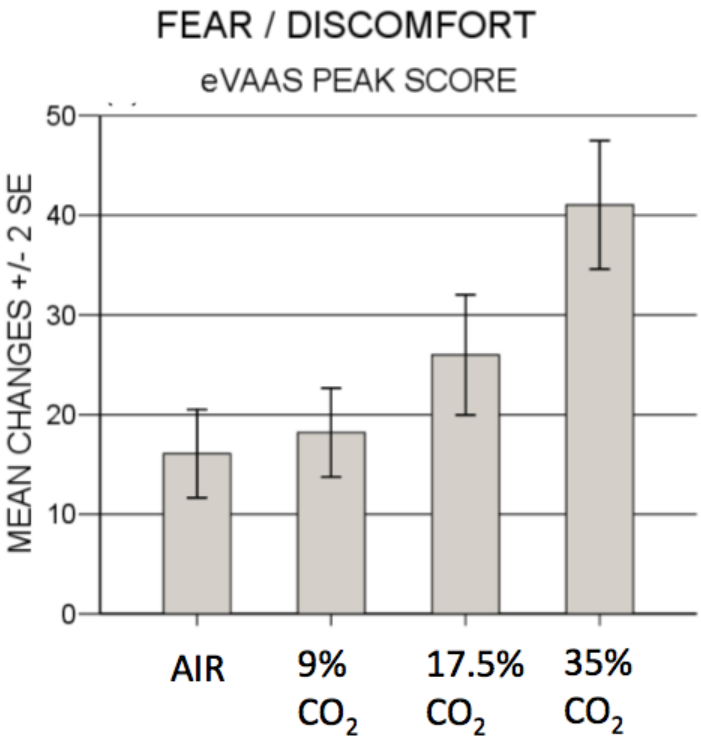
By definition, the mental state elicited in our studies is aroused by inhalation of gas mixture containing high concentration of CO_2. The type of stimulus is entirely sensorial; it has a strong motivational value, and correlate strongly with respiratory sensations (see Chapters 3 and 4).

Axis Y: **Intensity**

Definition*: Emotions are characterized by high intensity.*

We have observed that the feeling induced by CO_2 is dependent on the dose of CO_2 administered (see Fig. 1). There is a high inter-subject variability of the response. The peak intensity reported by the subjects receiving the highest dose (double breath 35% CO_2) reached the score of 50 (in a scale between 0 and 100 were 100 indicated the worst imaginable fear/discomfort) in more than 44% of the subjects.

Fig. 1) Dose-response relationship between CO_2 and induced fear/discomfort.



Axis Z: **Hedonicity**

Definition*: Emotions need to be highly hedonic, either positively or negatively.*

In our first study (see Chapter 2), we used a visual analogue scale labelled fear/discomfort, which identify a mental state with a negative valence. After the highest dose, 1 subject over 64 reported no increase from baseline, and 1 reported a decrease of 1 point score from baseline. Over 84% of subjects reported at least an increase of 10 from baseline after 35% CO_2. In our last study (see Chapter 5), we have assessed various affective dimensions of respiratory sensations induced by CO_2, and observed a significant increase relative to

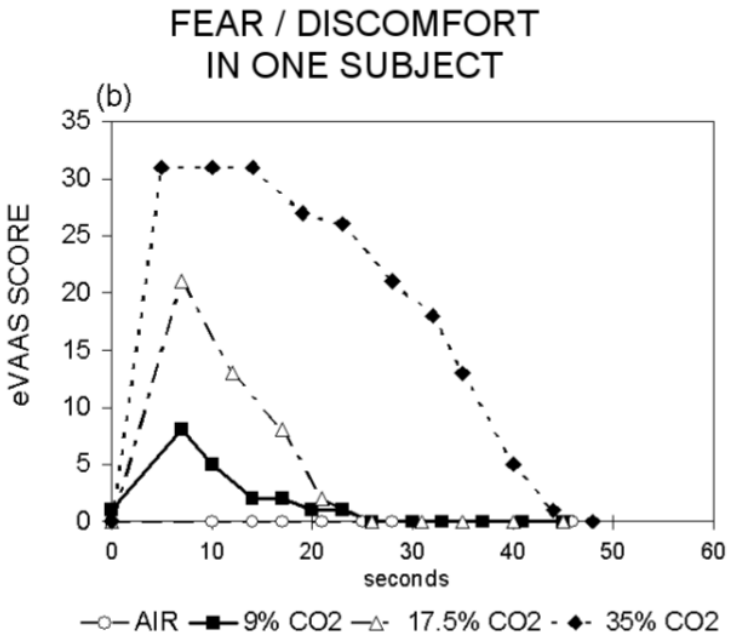
baseline in anxiety, fear and frustration ($p<0.0001$), anger ($p<0.001$), and depression ($p<0.05$), and a significant decrease in happiness ($p<0.0001$) and contentedness ($p<0.05$). Thus, the hedonic component of the mental state induced by CO_2 appears to be negative.

Axis T: **Duration**

Definition*: Emotion has a limited duration, closely related to the stimulus that aroused it.*

In the majority of the cases, the duration of CO_2-elicited mental state lasted less than one minute. The duration of the state was closely dependent on the dose administered (see Fig. 2).

Fig. 2) Time course of CO_2-induced fear/discomfort across different CO_2 doses.



In summary, the analysis of the four dimensions of emotions, according to Cabanac's definition, indicates that the subjective response observed in our studies:

1) *is a mental state elicited by a purely sensorial stimulus, CO_2 inhalation, which correlates with respiratory discomfort;*
2) *is characterized by an intensity which is dependent on the dose and becomes very intense with the highest CO_2 doses;*
3) *is characterized by high hedonicity with a negative valence;*
4) *is characterized by limited duration (less than 1 minute), related to CO_2 administration*.

In conclusion, the null hypothesis that the subjective response to CO_2 is merely the experience, or the perception, of a physiological reflex driven by interoceptors and is characterized by low intensity and low hedonicity, can be rejected, based on the evidence that administration of CO_2, at sufficiently high doses evokes a powerful mental state with a negative valence in a significant proportion of subjects. Hence, we are confident that CO_2 truly induced a genuine emotion.

Other views on primal emotions

Several influential authors have agreed about the existence of a type of emotions evoked by internal body states and whose evolutionary-based function is to motivate behaviour aimed at maintaining the body's internal balance. However, different names and definitions have been used to indicate these emotional states.

Denton's definition of primordial emotions has been already extensively discussed in the introduction (Denton, 2006). Other influential theories were proposed by Bud Craig, Antonio Damasio, and Jaak Panksepp.

Craig (Craig, 2003) focused his interest on the description of an anatomical pathway of afferents that innervate all tissues of the body, terminate monosynaptically in lamina I of the spinal and trigeminal dorsal horns, and conduct information regarding all types of

physiological conditions, including the mechanical, thermal, chemical, metabolic, and hormonal status of skin, muscle, joints, teeth, and viscera. According to Craig (2003), two separate pathways exist: a phylogenetically ancient one, and a more recently–evolved pathway, present in primates only. In all mammals, the projections from lamina I and the NTS pass through parabrachial nucleus and reach the anterior cingulate and insular cortices by way of the medial thalamic nuclei and the basal ventral medial nucleus of the thalamus. In humans and anthropoid primates instead, lamina I neurons and the NTS project directly to a specific thalamocortical relay nucleus in the posterolateral thalamus and eventually terminate to the interoceptive cortex in the posterior insula and the ACC. The direct activation of these structures by the distinct homeostatic modalities corresponds to the simultaneous generation of both a feeling/sensation dimension represented in the anterior insula and a motivation dimension represented in the ACC. As a consequence, a full homeostatic emotion arises. Craig believes these sites are activated in virtually every human emotions and provide an image of the physical self as a feeling (sentient) entity, which is a characteristic of human consciousness (Craig, 2003). He states that the subjective image of the 'material me' is formed on the basis of the sense of the homeostatic condition of each individual's body.

In his most recent book, Damasio (2010) acknowledges the existence of primordial feelings, which he defines as spontaneous reflections of the state of the living body, or images of an organism's internal state. These are supposedly based on the operation of upper-brain-stem nuclei and are part and parcel of the life-regulation machinery. These are feelings that tell the individual that his own body exists, and it is present, independently of any object with which it interacts. These have a definite quality and a valence and, according to the author, they constitute the basis of all feelings, including those caused by interactions between organism and external objects, which become the extension of the primordial feelings. Damasio proposes that primordial feelings are produced by the simplest stage of self-consciousness, which he defines "protoself" (Damasio, 1999, 2010).

Panksepp agrees with the existence of states related to homeostasis (Panksepp, 1998). He defines these as 'need' states, motivations, or regulatory urges. He distinguishes breathing regulation from other motivations as the needed resource, oxygen, under most circumstances is readily available. He acknowledges that when there is an impairment of rhythmic

breathing a regulatory crisis occurs, and a powerful emotional panic-like state arises. According to Panksepp (1998), other motivations such as the need for food or sex can be fulfilled only by an active exploratory and search behaviours, which are generated by an emotional system labelled ‘seeking system’, which can motivate organisms to pursue a diversity of distinct reward in the environment. However, it is not clear whether the author considers these states as real emotions. He acknowledges that they are affective feelings, however he states these does not fully qualify as emotions, as for instance, they do not fulfil the criterion that neural activity of all emotive systems is expected to outlast the precipitating circumstances.

Summary and concluding remarks

The findings reported in the present thesis demonstrate that CO_2 induces dose-dependent acute emotional distress in healthy volunteers, as measured with self-report subjective assessments. The distress resembles a panic attack when CO_2 doses are increased to an adequate level (Chapters 1 and 2). This is consistent with the notion of a human instinctual response to exposure of high concentrations of CO_2, with subjective elements which are phenomenologically similar to panic. Indeed, a principal component analysis showed that CO_2-induced symptoms cluster in dimensions analogous to the clusters of symptoms identified in clinical panic (Chapter 3).

A respiratory component was clearly identified among three extracted symptom dimensions. The respiratory dimension was the best correlate of subjective distress, indicating that the emotional response to CO_2 was specifically associated to respiratory sensations (Chapter 3). Furthermore, a multidimensional rating of respiratory discomfort was shown to be a stronger correlate of subjective distress compared to the rating of all non-respiratory panic symptoms. The level of respiratory discomfort could accurately discriminate between responders and non-responders to the CO_2 challenge (Chapter 4).

Finally, we observed that dietary manipulation of serotonin precursors influenced the affective response to CO_2, as lower availability of tryptophan was associated to lower subjective distress. The direction of the observed modulation appeared to be consistent with previously published preclinical data, indicating a role for the midbrain serotonergic system in promoting the aversive respiratory sensations to hypercapnic stimuli (Chapter 5). These findings, taken together with the recent discovery that the amygdala itself, a keystone in the affective brain, directly "senses" CO_2-released acidity, suggest that CO_2 plays an active role in the emotional regulation of the brain, and is not just a parameter of ventilatory control.

When reviewing his observations of behavioural vulnerability of patients with Panic Disorder, Klein proposed that panic attacks might be false biological alarms (Klein 1993) triggered by a 'suffocation detector' that is oversensitive. Our findings provide grounds for the existence of a hardwired inborn, CO_2 driven alarm machinery in humans. The sense of neuronal CO_2 loading, emerging into the conscious distress of panic to alert the organism

about the threat of imminent suffocation, fits perfectly with the idea that the affective response to CO_2 represents an instance of a primal emotion (Denton 2006).

The experimental paradigm presented in this thesis may represent a valid, reproducible, and safe empirical approach to study primal emotional systems in humans. Our work adds to the existing literature by stimulating a novel perspective on future CO_2–related affective neuroscientific research. In fact, we believe that the application of CO_2 challenges may go beyond the diagnostic boundaries set by DSM criteria for mental disorders. Studying the response to CO_2 may highlight not only the vulnerability to Panic Disorder, but in addition it could represent a tool to probe a primordial defensive response that is part of the human emotional repertoire. The application of our approach in neuropsychopharmacological research may be exciting: novel anxiolytic compunds could be assessed in very early phases of translation to clinical use on the basis of their ability to modulate response to CO_2 in healthy volunteers. Furthermore, psychopathology research would benefit from a dimensional approach based on the objective and reliable assessments of basic emotional systems, rather than solely relying on the current categorical/nosological paradigms.

It is attractive to speculate about the use of similar emotional challenges in the future. These could emerge as tools that can contribute towards a more personalized psychiatry, where pharmacological treatments are based on each individual characteristic, conceptualized as an ensemble of basic objectively-measurable biologically-rooted emotional traits, rather than focusing on diagnostic criteria based on overlapping syndromes of symptoms and aberrant behaviours. In other words, in our view the aim of testing primordial defensive responses in psychiatric patients would not be related primarily to diagnostic purposes, but instead it could be potentially useful to identify sensible biological targets for treatment at the individual level.

Further research on the biological mechanisms underlying primal emotional systems is needed before an application of models of CO_2-induced distress can be realised in translational medicine. Our work presented in this thesis and other recent studies have generated a set of new hypothesis that can be tested and potentially falsified. For instance, it would be important to test in humans the finding that limbic structures of the rodent brain

directly senses CO_2 released acidity (Ziemann et al. 2009). [^{31}P] magnetic resonance spectroscopy is an imaging technique that allows the in-vivo monitoring of regional brain pH. This technique is well suited to test if 1) CO_2 challenges truly induce a decrease in brain pH in the human brain and 2) if the subjective emotional response corresponds to the intensity of brain acidity. Another example of future potential research pertains to the investigation of synaptic neurotransmitters, in particular to answer the question which neurochemicals are released in response to CO_2 challenges. Molecular imaging with PET using radiotracers that are sensitive to displacement by endogenous neurotransmitters can be used to successfully detect and quantify the release of endogenous ligands at synaptic level, such as dopamine (Laruelle 2000) and opioids (Colasanti et al., 2012) in response to pharmacological challenges. These neurotransmitters, and in particular opioids, are promising candidates to try to modulate the brain response to CO_2 challenges due to the well-recognized link existing between CO_2, respiration and the opioid system, and between opioids and defensive behaviours (Colasanti et al. 2010). A PET study with [^{11}C]carfentanil is being planned to investigate the endogenous opioid response to CO_2 challenges.

Samenvatting en conclusies

De bevindingen beschreven in deze thesis demonstreren een dosis-afhankelijk effect van CO_2 op acuut emotioneel leed in gezonde proefpersonen. Het leed vertoont overeenkomst met die van een paniekaanval, wanneer CO_2 doseringen voldoende worden verhoogd (hoofdstuk 1 en 2). Dit is consistent met de veronderstelling dat er in mensen een instinctuele respons bestaat in antwoord op blootstelling aan hoge concentraties CO_2, en dat het subjectieve element van deze respons phenomenologisch gelijk is aan paniek. Een 'principal component analysis' toonde aan dat CO_2-geinduceerde symptomen clusteren in dimensies die analoog zijn aan het cluster van symptomen die gerelateerd zijn in klinische paniek (hoofdstuk 3).

Onder 3 ge-extraheerde symptoomdimensies werd duidelijk een respiratorische component geidentificeerd. De respiratorische dimensie correleerde het beste met de subjectieve maten van ongemak, wat suggereert dat de emotionele respons door CO_2 specific geassocieerd was met respiratorische sensaties (hoofdstuk 3). Bovendien was een multidimensionele beoordeling van respiratorisch ongemak een sterkere correlaat van de subjectieve ongemak, in vergelijking met de beoordeling van alle non-respiratorische panieksymptomen, en dit was in staat om accuraat een onderscheiding te maken tussen responders en nonresponders van de CO_2 toediening (hoofdstuk 4).

Tot besluit hebben we geobserveerd dat een diëetmanipulatie van de serotonine voorloper de affectieve respons van CO_2 beinvloedt, en dat de richting van de geobserveerde modulatie consistent blijkt te zijn met preklinische data, die aangaf dat het mid-brein serotonine systeem een rol speelt in het bevorderen van de aversieve respiratorische sensaties van hypercapnische stimuli (hoofdstuk 5). Deze bevindingen, samengenomen met de recente ontdekking dat de amygdala (een hoeksteen in het affectieve brein) zelf direct reageert op het zuur van CO_2, suggereert dat CO_2 een rol speelt in affective delen van het brein, en niet alleen een parameter is van ademhalingscontrole.

Naar aanleiding van een gedragsexperiment waarin de kwetsbaarheid van patienten met paniekstoornis naar voren kwam, stelde Klein voor dat een paniekaanval een biologische vorm van vals alarm is (Klein 1993) veroorzaakt door een overgevoelige benauwdheidsdetector. Onze bevindingen geven bewijs voor het bestaan van een aangeboren, CO_2-aangestuurd biologisch alarmsysteem. The sensatie van CO_2, voortvloeiend

in een bewust gevoel van paniek om het organisme te waarschuwen voor naderend zuurstofgebrek en verstikking, is uitstekend te verenigen met het idee van dat een affectieve respons op CO_2 een oer-emotie is (Denton 2006).

Het experimentele paradigma dat in deze thesis is gepresenteerd zou een valide, reproduceerbaar, en veilige empirische benadering kunnen bieden om oer-emotionele systemen in mensen te bestuderen. Ons werk verrijkt de bestaande literatuur door een nieuw perspectief te bieden op toekomstig CO_2-gerelateerd affectief neurowetenschappelijk onderzoek. Metterdaad zijn we ervan overtuigd dat de toepassing van CO_2 toedieningen de grenzen kan overschrijden van de DSM criteria voor mentale stoornissen. Door de respons van CO_2 te bestuderen zou men niet alleen de kwetsbaarheid voor paniekstoornissen kunnen uitlichten, maar bovendien een methode bieden om evolutionair gezien oude verdedigingsmechanismen te onderzoeken die deel uitmaken van het repertoire van menselijke emoties. De toepassing van onze benadering in neuropsychopharmacologisch onderzoek heeft potentieel: nieuwe psychoactieve stoffen zouden kunnen worden geevalueerd in de vroege fase van de translatie naar klinisch gebruik op basis van hun mogelijkheid om de respons op CO_2 in gezonde proefpersonen te moduleren. Bovendien zou psychopathologisch onderzoek baat hebben bij een dimensionele benadering gebaseerd op objectieve en betrouwbare beoordelingen van basale emotionele systemen, in plaats van voornamelijk afhankelijk te zijn van de huidige categorische/nosologische paradigma's.

Het is aantrekkelijk om te speculeren over het gebruik van gelijkwaardige emotionele challenges in de toekomst, als middelen die kunnen contribueren aan een meer persoonsgebonden psychiatrie, waar pharmacologische behandelingen gebaseerd zijn op elke individuele eigenschap, geconceptualiseerd als een bijeenvoeging van basale objectief meetbare, biologisch diepgewortelde emotionele trekken, geen te focussen op diagnostische criteria die gebaseerd zijn op overlappende syndromen van symptomen en afwijkend gedrag. Met andere woorden, het doel van het testen van verdedigingsmechanismen in psychiatrische patienten zou niet primair gericht zijn op diagnostiek, maar om potentiele biologische doelen te identificeren voor behandeling op individueel niveau.

Vervolgonderzoek is nodig om de biologische mechanismen van basale emotionele systemen te verduidelijken, voordat toepassing van CO_2-geinduceerd ongemak kan worden gerealiseerd. Ons werk, en ander recent onderzoek, heeft een nieuwe set hypotheses gegenereerd die kunnen worden getest en eventueel kunnen worden gefalsificeerd. Het zou bijvoorbeeld belangrijk zijjn om de preclinische bevinden te testen in mensen, specifiek de hypothese dat de limbische structuren van het brein direct een CO_2 vrijgegeven zuur kunnen 'voelen'. ^{31}P MRS is een nuttige beeldvoormende techniek waarmee in-vivo de regionale pH in de hersen kan worden gemeten. Het zou waardevol zijn om te testen of 1) CO_2 toediening inderdaad een daling van pH in het menselijk brein veroorzaken en 2) of the emotionele respons correspondeert met de intensiteit van de zuurgraad. Een ander voorbeeld van potentieel toekomstig onderzoek betreft de vraag welke synaptische neurotransmitters worden vrijgegeven na CO_2 toediening. Moleculaire beeldvoormende technieken zoals PET met radioliganden die sensitief genoeg zijn om de verplaatsing van endogene neurotransmitters van de receptor te detecteren en quantificeren zouden bijvoorbeeld dopamine (Laruelle 2000) of opioiden (Colasanti et al, 2012) niveaus kunnen meten na farmacologische challenge. Deze neurotransmitters, en vooral opioiden, zijn veelbelovende mechanismen in het brein om te moduleren na toediening van CO_2. Dit borduurt voort op eerder gepubliceerd onderzoek waarin we een link hebben aangetoond tussen CO_2, ademhaling en het opioid systeem, en tussen opioids een verdedigend gedrag (Colasanti et al, 2010). Een PET experiment met [^{11}C]carfentanil is gepland om endogene opioids te bestuderen na toediening van CO_2.

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Curriculum Vitae

Alessandro Colasanti was born on March 22, 1979 in Milan, Italy. He enrolled in medical school in 1999 in Milan. He spent his fifth medical school year at Maastricht University in the context of the Erasmus student exchange programme. In 2003, he began research work at the Section of Experimental Psychiatry, Maastricht University, under Professor Griez's supervision. He then completed the medical school and obtained the Medical Degree at the University of Milan in 2004, and began specialist training in psychiatry immediately afterwards. Over the course of the specialist training, he undertook the International Master in Affective Neuroscience from Maastricht University and University of Florence, and graduated in 2007. In 2008 he completed the specialist training in Psychiatry at University of Milan and returned to Maastricht to complete his research work. In 2009 he has been awarded the ECNP grant for young scientists and moved to Imperial College of London. In 2010, he was awarded a 3 years Wellcome Trust training fellowship in Translational Medicine and Therapeutics. He is currently a Clinical Research Fellow at the Division of Brain Sciences at Imperial College of London working on Positron Tomography Imaging research projects focused on the study of the endogenous opioid system and neuroinflammatory processes in the living human brain.

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